



FACULTY OF VETERINARY MEDICINE  
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# Quantification of the *Campylobacter* contamination of broiler carcasses during slaughter

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Thesis submitted in fulfillment of the requirements  
for the degree of Doctor (PhD) in Veterinary Sciences

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## **GENERAL INTRODUCTION**

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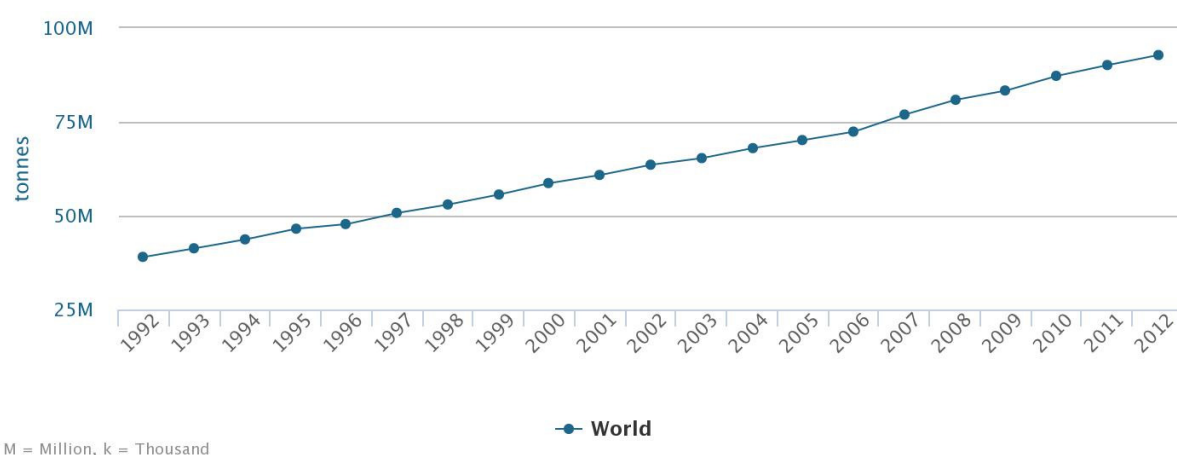


## 1. Poultry meat

According to the Regulation (EC) No 853/2004 (Anonymous, 2004a) poultry is defined as farmed birds, including birds that are not considered as domestic but which are farmed as domestic animals, with the exception of ratites. Meat means edible parts of the animals including blood and fresh meat has not undergone any preserving process other than chilling, freezing or quick-freezing, including meat that is vacuum-wrapped or wrapped in a controlled atmosphere (Anonymous, 2004a).

### 1.1. Poultry meat demand

Over the last years rapid growth in livestock production has been observed and poultry has the major contribution to the livestock production increase (Thornton, 2010). Globally, annual poultry meat production grew by more than 50 million tons between 1992 and 2012 (Fig. 1.1). This trend has been caused by rising poultry meat demand driven by increasing population, higher incomes, diversification of diets and expanding markets. Moreover, it is estimated that poultry meat production will rise additionally by 28.8 million tons during the period 2014-2023 representing almost 50 % of the additional production of all kinds of meats (AVEC, 2014). The increase in poultry meat production has been most evident in East and Southeast Asia and in Latin America, particularly in China and Brazil. In Belgium, poultry production increased slower in comparison to the world trend and accounts for 499 313 tons in 2010, whereas in 2004, 460 611 tons of poultry meat were produced (VLAM 2014).



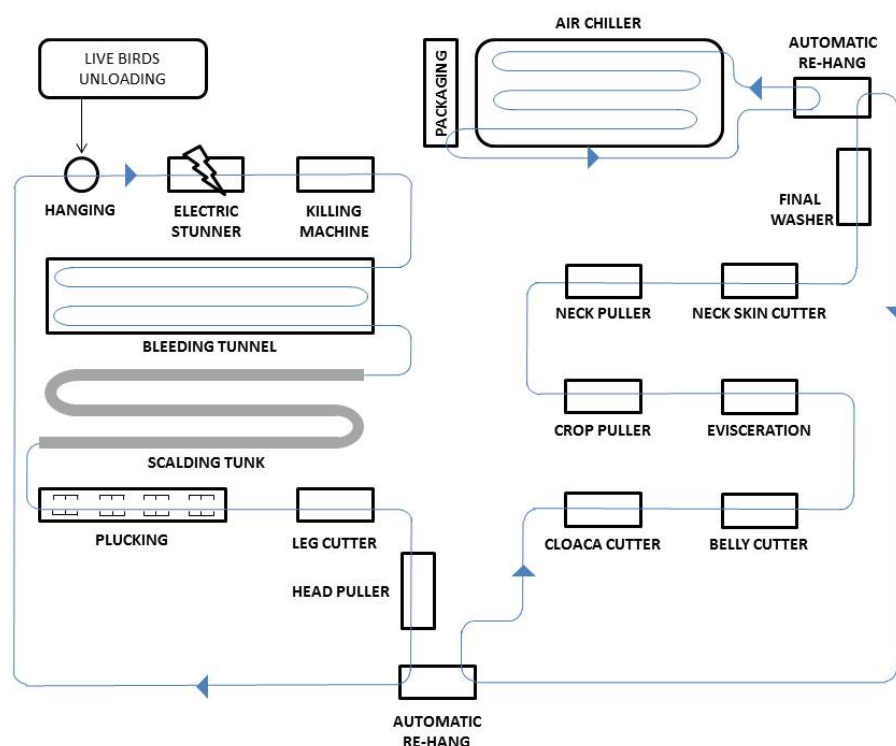
**Figure 1.1. Global chicken meat production in years 1992 - 2012 (Source: <http://faostat3.fao.org/>)**

Various poultry species, but mainly chickens (*Gallus gallus*), are reared in the industrial poultry meat production, and their importance varies with regions and food consumption habits (EFSA, 2011a). In Belgium most of consumed poultry meat (more than 80 %) is represented by chicken meat and therefore

the focus of this thesis will be primarily on this species. In 2013 an annual consumption of broiler meat was reported as 8.1 kg/capita, encompassing 7 and 1.1 kg of fresh and frozen meat, respectively (VLAM 2014).

## 1.2. Broiler meat production chain

Most birds are raised in closed systems, inside broiler houses with a maximum stocking density of 33 kg/m<sup>2</sup> (Anonymous, 2007). Broiler flocks in Belgium, encompassing between 9 and 60 thousands birds, are collected on the farm at an age of 35 or 42 days, placed into crates and transported to the processing plant. Next, broilers are processed on the same day in fully automated slaughterhouses with the production capacity range between 6 and 12 thousand chickens per hour. Fig. 1.2 presents a schematic flow chart of the slaughter process.



**Figure 1.2 Schematic flow chart of one of selected Belgian slaughterhouse**

Broilers are unloaded, hanged on hooks and stunned with electricity in a water bath. If the gas stunning is applied, birds are stunned before hanging. The main advantage of gas stunning is an increased animal welfare (*i.e.* no stress of shacking, avoiding pre-stun electric shocks, no possibility of different current, no possibility of missing the stunning procedure). Gas stunning as well as electric stunning according



annex I of Regulation (EC) No 1099/2009 (Anonymous, 2009) kills most of the birds what prolongs the bleeding.

Next, stunned birds are killed automatically by incision of the neck with motor driven rotating knife. After bleeding out, broilers are scalded at approximately 53 °C to open the feather follicles to facilitate the removal of feathers during plucking. During plucking, carcasses pass through the plucking machine equipped with rubber, plucking fingers on rotating disks. After removing the head and feet, the cloaca is opened and the viscera is separated from the carcass during the evisceration process. Next, crop is removed by the rotating drill that goes through the carcass. Before air chilling, carcasses are finally washed in the inside-outside washer with the potable water. In an inside-outside carcass washer a probe with a single spray nozzle enters the bird's intestinal cavity and washes the inside. Then a series of spray nozzles wash the entire outside of the bird (Keener et al., 2004).

### **1.3. Public health concern for consumption of poultry meat**

Increasing poultry meat consumption elevates public health concern about chemical and biological hazards related to poultry meat. Regarding chemical hazards, dioxins, dioxin-like polychlorinated biphenyls (DL-PCBs), and the banned antibiotics chloramphenicol, nitrofurans and nitroimidazoles were ranked as being of high potential concern. However, chemical substances in poultry are unlikely to pose an acute health risk for consumers (EFSA, 2012a).

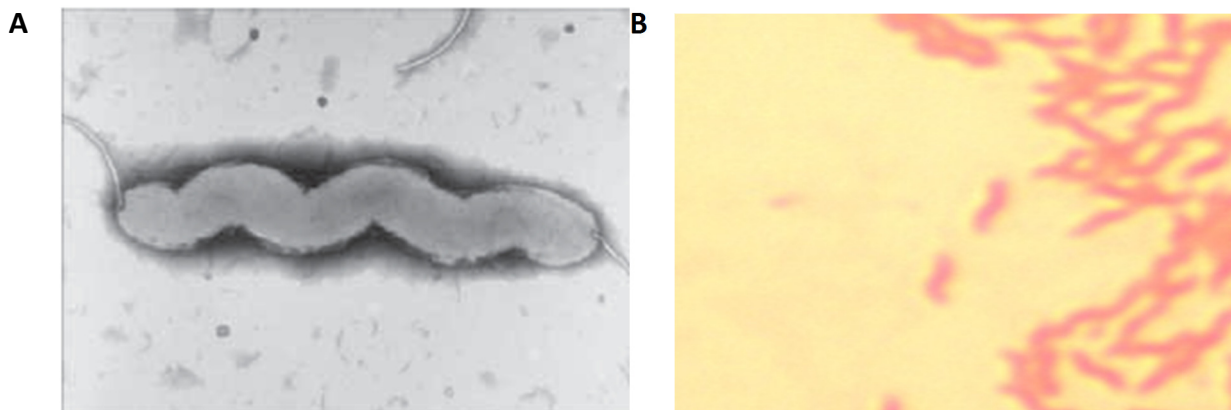
Based on the magnitude of the human health impact (*i.e.* the severity of the disease in humans; the proportion of human cases that can be attributed to the handling, preparation and consumption of poultry meat; and the occurrence of the hazards in poultry flocks and carcasses) *Campylobacter* and *Salmonella* were considered to be of high public health relevance followed by extended-spectrum  $\beta$ -lactamase (ESBL)/AmpC gene-carrying *E.coli* (EFSA, 2012a). Present thesis, will focus on *Campylobacter* as the most frequently reported zoonosis in EU.

## **2. *Campylobacter* species**

As early as 1886, Theodor Escherich described nonculturable spiral shaped bacteria. For the first time, *Campylobacter* (at that time called *vibrios*) was isolated by McFadyean and Stockman in 1913 from aborted ovine fetuses. Genus *Campylobacter* was established in 1963 by Sebald and Veron although only in 1970s, thanks to improved isolation methods, *C. jejuni* and *C. coli* have been identified as an important cause of human enteric illness (Butzler et al., 1973; Skirrow, 1977).

*Campylobacter* spp. comprise small (0.2-0.9  $\mu\text{m}$  wide and 0.2-5.0  $\mu\text{m}$  long), spiral formed (Fig. 1.3), Gram-negative bacteria. Their motility is achieved by the presence of a single polar flagellum present at

either one or both ends of the cell which gives the organism its characteristic corkscrew-like movement. *Campylobacter* spp. do not form spores although cells in old cultures may form spherical or coccoid bodies (Moran and Upton, 1987).



**Figure 1.3. Electron micrograph of *Campylobacter jejuni* (Gaynor et al., 2004) (A); *Campylobacter* after coloration with safranin; picture was kindly donated by Dr. Julie Baré (B).**

The *Campylobacter* genus is continually growing with currently 25 species assigned to the Genus *Campylobacter* (Table 1.1). Among the campylobacters *Campylobacter jejuni* and *Campylobacter coli* are the most important human enteropathogens, accounting for approximately 80 and 6 % of all human cases in EU during 2012, respectively. Based on the European baseline study *C. jejuni* and *C. coli* are also highly prevalent in broilers. On average *C. jejuni* was detected in 40.6 % of tested samples, whereas *C. coli* in 31.9 %. Occasionally *C. lari* can be found in broilers although at the prevalence lower than 1 % (EFSA, 2010a). The prevalence of *Campylobacter* species differs between EU members. In northern countries (Estonia, Finland, Sweden and Norway) *C. coli* was not detected and *C. jejuni* is in general more prevalent in this part of Europe. In contrast, the highest (> 50 %) *C. coli* prevalence was noted in southern Europe (e.g. Malta, Spain and Portugal) (EFSA, 2010a).

**Table 1.1. Species and subspecies assigned to the genus *Campylobacter*.****(Source: <http://www.bacterio.net/campylobacter.html> and Debruyne et al., 2008)**

No.	Species	Reference	Main recognized host(s)
1.	<i>Campylobacter avium</i>	Rossi et al., 2009	Caecal contents of broiler chickens
2.	<i>Campylobacter canadensis</i>	Inglis et al, 2007	Cloaca of whooping cranes
3.	<b><i>Campylobacter coli</i></b>	Doyle, 1948	First isolated from faeces of pigs
4.	<b><i>Campylobacter concisus</i></b>	Tanner et al., 1981	Humans
5.	<i>Campylobacter corcagiensis</i>	Koziel et al., 2014	Lion-tailed macaques
6.	<i>Campylobacter cuniculorum</i>	Zanoni et al., 2009	Rabbits
7.	<i>Campylobacter curvus</i>	Tanner et al., 1984	Humans
8.	<b><i>Campylobacter fetus</i></b>	Smith and Taylor, 1919	E.g. ungulates, fowl, reptiles, humans
9.	<b><i>Campylobacter gracilis</i></b>	Tanner et al., 1981	Gingival crevices in humans
10.	<i>Campylobacter helveticus</i>	Stanley et al., 1993	Domestic animals
11.	<b><i>Campylobacter hominis</i></b>	Lawson et al., 2001	Humans
12.	<b><i>Campylobacter hyointestinalis</i></b> <sup>a</sup>	Gebhart et al., 1995	Pigs, hamsters, cattle, deer, humans
13.	<i>Campylobacter insulaenigrae</i>	Foster et al., 2004	Marine mammals
14.	<b><i>Campylobacter jejuni</i></b>	Véron & Chatelain, 1973	Intestinal tract of birds
15.	<i>Campylobacter lanienae</i>	Logan et al., 2000	Pigs, bovine, humans
16.	<b><i>Campylobacter lari</i></b>	Benjamin et al., 1983	Seagulls, river water, shellfish
17.	<i>Campylobacter mucosalis</i>	Roop et al., 1985	Intestinal mucosa of pigs
18.	<i>Campylobacter peloridus</i>	Debruyne et al., 2009	Humans and molluscs
19.	<b><i>Campylobacter rectus</i></b>	Tanner et al., 1981	Gingival crevices of humans
20.	<b><i>Campylobacter showae</i></b>	Etoh et al., 1993	Human dental plaque
21.	<b><i>Campylobacter sputorum</i></b>	Véron & Chatelain, 1973	Humans, bulls, sheep, pigs, cattle
22.	<i>Campylobacter subantarcticus</i>	Debruyne et al., 2010a	Birds in the sub-Antarctic region
23.	<b><i>Campylobacter upsaliensis</i></b>	Sandstedt & Ursing, 1991	Humans, dogs, cats, and meerkats
24.	<b><i>Campylobacter ureolyticus</i></b>	Vandamme et al., 2010	Humans
25.	<i>Campylobacter volucris</i>	Debruyne et al., 2010b	Black-headed gulls

<sup>a</sup>subsp. *hyointestinalis* in children and HIV patients

Species in bolded letters play a pathogenic role in humans.

## 2.1. Growth conditions and survival in the food chain

*Campylobacter* spp. are essentially micro-aerophilic organisms, growing best in an atmosphere containing approximately 10 % CO<sub>2</sub>, 5 % O<sub>2</sub> and 85 % N<sub>2</sub>. Pathogenic species are able to grow in a temperature range of 37-42 °C with an optimum at 41.5 °C (body temperature of birds) and they are classified as thermophilic campylobacters (Humphrey et al., 2007). Important from a food safety and public health perspective, these demanding growth conditions imply that *Campylobacter* can only multiply in the intestinal tract of warm-blooded animals but not in the foodstuff, therefore (Wagenaar et al., 2013).

*Campylobacter* is exposed to many stresses during food processing, in particular low and high temperatures but also the osmotic shock, drying, oxidative stress and acid conditions.

It is still debatable how sensitive *Campylobacter* is to conditions outside the host. It has been reported that freezing can significantly reduce *Campylobacter* counts during the first 24 hours what is related to the cells damage. According to Archer (2004) this damage might be expected from the following factors: (1) extracellular ice formation, (2) intracellular ice formation, (3) concentration of extracellular solutes, (4) concentration of intracellular solutes and (5) low temperature. In addition, there is evidence that oxidative damage has also been implicated as a cause of cellular injury during freezing (Stead and Park, 2000).

Further decrease in counts during the prolonged storage at  $-22^{\circ}\text{C}$  is slow and graduate (Georgsson et al., 2006; Sampers et al., 2010a). Under refrigeration conditions ( $4^{\circ}\text{C}$ ), *Campylobacter* can survive extended periods on food products such as raw chicken (Pintar et al., 2007) and even it has been reported that *C. jejuni* survives better at  $4^{\circ}\text{C}$  than at  $22$  or  $30^{\circ}\text{C}$  (Kelana and Griffiths, 2003).

*Campylobacter* are also relatively sensitive to heat and broiler meat exposure to the frying process can inactivate naturally occurring *Campylobacter* if the internal temperature reaches  $70^{\circ}\text{C}$  (Sampers et al., 2010a; Whyte et al., 2006).

Although *Campylobacter* is a micro-aerophilic organism that need to cope with aerobic stress, it is able to survive under aerobic exposure (Flint et al., 2014; Habib et al., 2010). Furthermore, sensitivity of *Campylobacter* to acids, irradiation and desiccation has been shown. In order to survive under these conditions *Campylobacter* developed an adaptive tolerance response (i.e. induces an adaptive response to sub-lethal stress and provides protection towards subsequent exposure to a lethal stress) or found ecological niches for survival. For example it was documented that chicken skin texture that protects *Campylobacter* from drying (Murphy et al., 2006). Additionally, chicken skin can contain protective chemical components, such as proteins, fatty acids, and oils that may enhance the survival of contaminating bacteria (Lee et al., 1998). Although the majority of *Campylobacter* cells remained on the skin surface, they have been also located at the bottom of feather follicles and deep channels in chicken skin providing a suitable microenvironment for the survival of *Campylobacter* (Chantarapanont et al., 2003). There is also evidence of taxis of *Campylobacter* on skin surfaces into skin follicles under aerobic conditions to escape an oxygen environment (Jang et al., 2007).

Another common strategy for bacterial survival in stressed conditions is biofilm formation. It has been demonstrated that *Campylobacter* can form biofilm in low nutrient or aerobic conditions which later can figure as a reservoir of viable cells possibly contributing to the infection (Reeser et al., 2007; Reuter et al., 2010)

Under prolonged periods of starvation or presence of oxygen *Campylobacter* can enter the viable but non-culturable (VBNC) state (Murphy et al., 2006). *Campylobacter* in this state lose the ability to form colonies and cannot be grown using routine culture based analyzes. However, *Campylobacter* can

recover from the VBNC state under favorable conditions and pose the risk of infection (Baffone et al., 2006).

Moreover, there are evidences of the beneficial interaction of *Campylobacter* with other microorganism that allow *Campylobacter* to survive outside the host. According to Teh et al. (2010), mixed species communities promote *Campylobacter* biofilm growth. Additionally, *Campylobacter* can survive longer under oxidative stress when it is co-cultured with *Pseudomonas* spp. (Hilbert et al., 2010). It has been also shown that co-culture with *Acanthamoeba* can increase long-term survival of *Campylobacter* although compelling evidence that protozoa represent a potential reservoir for *C. jejuni* in natural environments is lacking (Baré et al., 2010).

Despite the fact that *Campylobacter* is considered to be more sensitive in comparison to other foodborne pathogens, it was presented that it has abilities to survive in food in sufficient numbers to cause infection in humans.

### 3. Campylobacteriosis

Most *Campylobacter* infections in humans are caused by *C. jejuni* and *C. coli* although in the developing countries *C. upsaliensis* is also important (Humphrey et al., 2007). The infection dose of campylobacteriosis is considered low at a few hundred cells (Black et al., 1988). Various virulence factors in campylobacters contribute to the survival and the establishment of disease in the host. First, due to *Campylobacter* motility target area within the gastrointestinal tract can be reached (Guerry, 2007).

Additionally, it has been suggested that flagellum may also be involved in adherence to epithelial cells and therefore the flagellum is an essential virulence factor for *Campylobacter* (Grant et al., 1993). Also chemotaxis, is essential for *Campylobacter* infections. Bacterial chemotaxis is a complex signal transduction system by which bacteria are able to react on the chemical gradients and respond to them by flagellar rotation. *Campylobacter jejuni* is attracted to mucins, L-serine and L-fucose, whereas bile acids are repellants (van Vliet and Ketley, 2001). Important aspects of *Campylobacter* infection are adhesion and invasion of host cells. The invasion of epithelial cells results in inflammation which may have a role in the development of diarrhea (van Vliet and Ketley, 2001). It has been presented that *Campylobacter* can produce cytolethal distending toxin that contributes to its pathogenicity (Wassenaar, 1997). A major problem for *C. jejuni* in establishing infection is the availability of free iron in host fluids and therefore the ability of pathogenic bacteria to acquire iron is important for establishing infection (Bhavsar and Kapadnis, 2006). Additionally as reviewed by (van Vliet and Ketley, 2001), the outer membrane constituents lipo-oligosaccharide (LOS) and lipopolysaccharide (LPS) form a major component of the Gram-negative outer membrane, and are important virulence factors involved in serum resistance, endotoxicity and adhesion. It has been also suggested, that surface polysaccharide

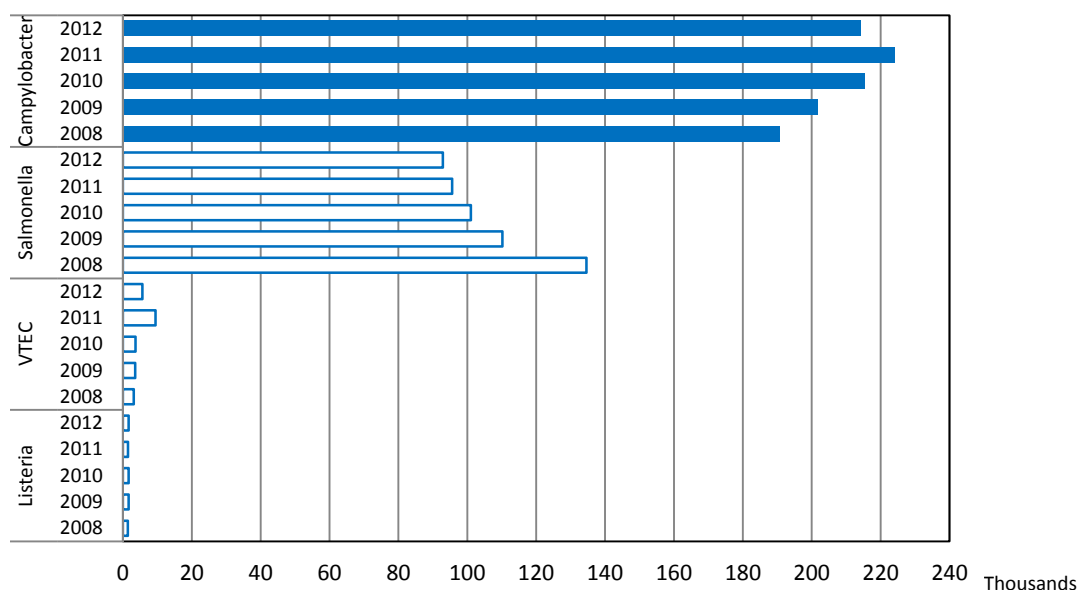
structures is involved in ganglioside mimicry leading to Guillain-Barré and Miller Fisher syndrome (Houliston et al., 2007). In order to survive in the host, *Campylobacter* developed oxidative and thermal stress defense systems. These abilities allow *Campylobacter* to answer to toxic oxygen metabolites and adjust to the host temperature and they support *Campylobacter* survival during transmission in food chain production (van Vliet and Ketley, 2001). The virulence mechanisms of *Campylobacter* discussed above showed the mode of infection in the human body and its survival strategies leading to human gastroenteritis and sometimes also to post-infectious complications.

### **3.1. Symptoms**

Following exposure, *C. jejuni* colonizes the lower intestinal tract (ileum, jejunum, and colon) often without causing any symptoms (Dasti et al., 2010). In symptomatic cases, the most prominent clinical manifestation of campylobacteriosis in humans is gastroenteritis. The disease is accompanied with abdominal cramps, shortly followed by diarrhea. Other symptoms in order of decreasing frequency are fever, headache, myalgia, blood in feces, and vomiting (Blaser and Engberg, 2008). The illness usually lasts one week, although it can also cause post-infectious complications. Approximately 1 in 1000 patients develops an autoimmune-mediated neurological disorder called Guillain-Barré syndrome (Nachamkin, 2002). Additionally, campylobacteriosis have been associated with the development of Miller Fisher syndrome (Lee, 2012), reactive arthritis (Hannu et al., 2002), irritable bowel syndrome (Gradel et al., 2009) and possibly inflammatory bowel disease (Havelaar et al., 2009). In 2013, 31 deaths due to campylobacteriosis were reported what results in an EU case-fatality rate of 0.05 % (EFSA, 2015) being the lowest among zoonoses listed in Figure 1.4.

### **3.2. Incidence rate and disease burden**

Since its recognition as a human pathogen in the early 1970s, *Campylobacter* has now emerged as the leading bacterial cause of food-borne gastroenteritis (Fig 1.3). In 2013, the number of reported confirmed cases of human campylobacteriosis in the EU was 214 779 and the EU notification rate account for 64.80 per 100,000 population(EFSA, 2015).

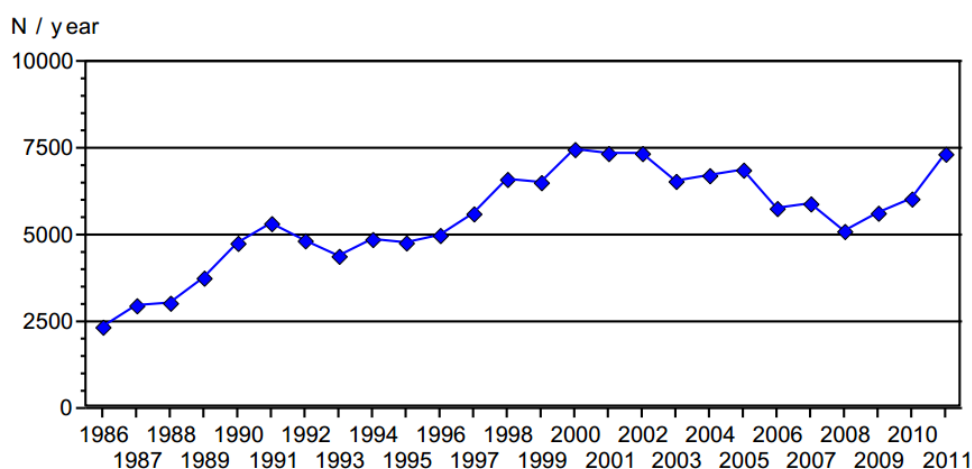


**Figure 1.4. Reported cases of selected zoonoses in humans in EU (2008 – 2012). Source: EFSA**

Similar trend was observed in Belgium, where since 2005 campylobacteriosis remains the most frequently reported zoonosis in humans. Additionally, the number of *Campylobacter* infections shows a significant increase trend since 2008 and reach the number of 7301 confirmed cases in 2011 (Fig. 1.5). Belgian notification rate account for 68 per 100,000 population in 2011 (FASFC, 2012).

As symptoms of campylobacteriosis are self-limited, people rarely consult doctor or patients are not requested to provide a stool sample. Therefore, it is hard to define true incident rate of campylobacteriosis. It is estimated that only 1/47 campylobacteriosis cases is reported on average in EU and 1/11 in Belgium (Havelaar et al., 2012).

When taking into account these incidence estimates, the public health impact is then calculated at 0.35 million disability-adjusted life years (DALYs) per year for the EU with an annual cost of about 2.4 billion € (EFSA, 2011a).



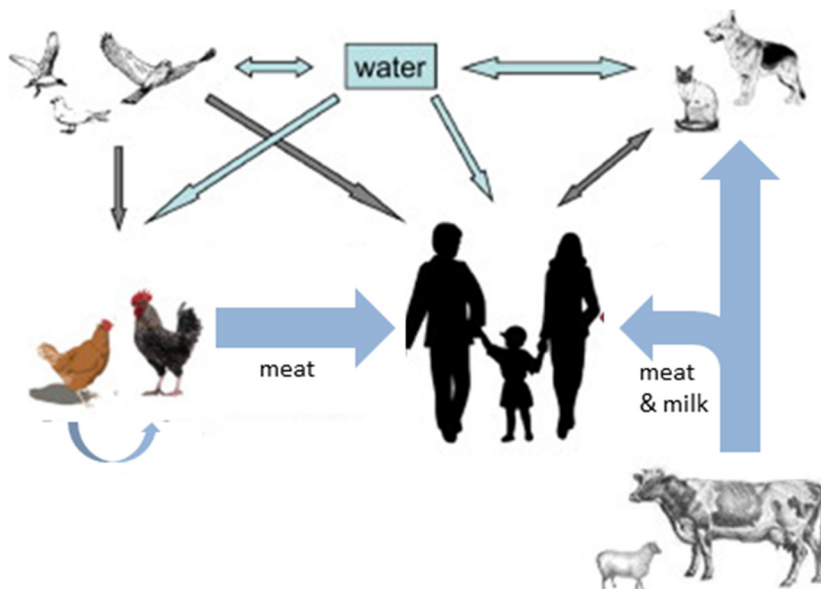
**Figure 1.5. Number of *Campylobacter* infections in humans by year (1986–2011) in Belgium. Source: <http://www.afsca.be>**

There was a clear seasonal trend in confirmed campylobacteriosis cases during the summer months (EFSA, 2015). This could be explained by a variation in human behavior, such as increased animal contact, eating barbecue meals, drinking or accidental ingestion of untreated water (Newell et al., 2011). Additionally, Christensen et al. (2005) demonstrated that gender and age play a role in risk exposure.

### 3.3. Sources of *Campylobacter* infections in humans

Source attribution studies indicate a definite link between human infection strains and poultry isolates (Boysen et al., 2013; Strachan et al., 2012). It is estimated that the majority (50 - 80%) of strains infecting humans originate from the chicken reservoir as a whole, whereas handling and consumption of broiler meat may account for 20 to 30 % of all human campylobacteriosis cases and EFSA (2011a). These findings suggest that strains from the chicken reservoir may also reach humans by pathways other than food (e.g. via the environment or by direct contact) (Fig 1.6).

Except for the poultry meat, outbreaks related to the consumption of raw cow's milk, contaminated water and raw vegetables have been reported (Bartholomew et al., 2014; Gardner et al., 2011; Heuvelink et al., 2009). Based on a meta-analysis study consumption of beef, pork and lamb comprise low risk of infection (Domingues et al., 2012).



**Figure 1.6. Important routes for human infection by *Campylobacter*. Figure adopted from Dasti et al. (2010) and modified.**



## 4. *Campylobacter* in broiler meat chain production

### 4.1. *Campylobacter* at the farm level

#### 4.1.1. *Campylobacter* in broilers

*Campylobacter* is highly prevalent in broilers. The EU baseline survey showed that at the average prevalence of *Campylobacter* colonization in broiler flocks, as determined from caecal contents, was 71.2%. However, proportion of *Campylobacter* positive flocks varies between participating European countries, and ranged between 2 and 100 % for Estonia and Luxemburg, respectively. *Campylobacter* prevalence at the level of 31 % classified Belgium below the European average. Such differences between countries might be partially reflected by the climate *characteristics*. Additionally, it is worth mentioning, that countries like for example Denmark, Norway and Iceland successfully implemented *Campylobacter* control programs, what may result in lower *Campylobacter* prevalence (EFSA, 2010a).

In general, it is assumed that birds carrying *Campylobacter* are asymptomatic colonizers without any clinical signs (Dhillon et al., 2006) and it is thought that *Campylobacter* is part of the normal microbiota of birds. However, more recent study (Humphrey et al., 2014), shows that modern rapidly growing chickens used in intensive production systems have a strong inflammatory response to *Campylobacter* infection, resulting in diarrhea, which, in turn, leads to damage to the feet and legs on the birds due to standing on wet litter. Shown evidences that *Campylobacter* colonization influence animal health can encourage farmers to improve their biosecurity and general production hygiene.

#### 4.1.2. Vertical *Campylobacter* transmission

The role of vertical transmission as a flock colonization source is still debated. Breeder hens are usually colonized by *Campylobacter* (Petersen et al., 2001b), and this bacterium was also detected in the rooster semen as well as in the reproductive tract of breeder hens (Hiett et al., 2002; Vizzier-Thaxton et al., 2006). Additionally, *Campylobacter* was detected by qPCR, but not by the culture method, in embryos from breeder hens (Rossi et al., 2012). Based on these evidences, transmission from breeder hens to broiler chickens has been suggested.

Conflicting reports have also been published indicating that vertical transmission is unlikely or of little importance. Sahin et al. (2003) did not detect *Campylobacter* in a large number of eggs (shell surface and content) from commercial breeders that were *Campylobacter* positive. Consequently, *Campylobacter* vertical transmission to broiler offspring through the egg might be a rare event. Additionally, breeder flocks were found to be colonized with different *flaA*-SVR and ST *Campylobacter*

types than related broiler flocks (Callicott et al., 2006; O'Mahony et al., 2011). This reveals that vertical transmission is probably not involved in broiler flock colonization.

While the debate about possible vertical transmission continues, control of horizontal *Campylobacter* colonization sources seems to be more pragmatic approach in *Campylobacter* prevention (Newell and Fearnley, 2003).

#### 4.1.3. Sources of *Campylobacter* horizontal transmission

Although *Campylobacter* is highly prevalent in poultry production systems, the exact routes of poultry flocks infection is still unknown. Given the widespread of *Campylobacter* in the farm environment, it is not surprising that numerous transmission factors for broiler colonization with *Campylobacter* have been reported in the literature (Table 1.2).

The most common source of broiler flock colonization is other livestock present at the farm or in the close neighborhood. The same *Campylobacter* genotype was mostly shared between broiler flock and neighboring poultry or cattle (Johnsen et al., 2006; O'Mahony et al., 2011; Ridley et al., 2011; Zweifel et al., 2008). However, as suggested by Agunos et al. (2014) more data is needed to confirm directionality of *Campylobacter* spread between species at the farm. Possibly infected broiler flock might be a *Campylobacter* source for the other animals. Similarly, Hald et al. (2004) and Bates et al. (2004) found common *Campylobacter* types between infected broiler flocks and insects but there is still no prove that broiler flocks were infected by contact with insects or if the order was reversed.

Common on farm wild birds were also examined for the *Campylobacter* presence and the average prevalence reviewed by Agunos et al. (2014) account for 37.2 %. Messens et al. (2009) have reported relationship between *Campylobacter* types in wild birds and in broiler flocks. However, in the other study the similarity between genotypes detected in broiler flocks and wild geese was low (Colles et al., 2008). This finding is supported by Petersen et al. (2001) who no significant relationship between wildlife strains and human/chicken strains. This suggests limiting importance of wildlife as a reservoir of infection.

**Table 1.2 Potential sources of broilers infection based on molecular epidemiology evidences**

Source	Description	References
<u>Animal source</u>		
Other flock	The same <i>Campylobacter</i> type was isolated from adjacent broiler flocks and investigated broiler flock.	(O'Mahony et al., 2011)
Other broiler farm	The same AFLP type was found from broilers at a neighbor farm 2 weeks prior to the infection of selected flock.	(Johnsen et al., 2006)
Laying hens	Certain <i>Campylobacter</i> genotypes ( <i>flaA</i> -RFLP and PFGE) persisting in laying hens were found in broiler flock.	(Zweifel et al., 2008)
Cattle	PFGE patterns showing matches between cattle and broiler faeces.	(Ridley et al., 2011).
Pig	Certain <i>Campylobacter</i> genotypes ( <i>flaA</i> -RFLP and PFGE) persisting in laying hens were found in broiler flock.	(Zweifel et al., 2008)
Flies	The same PFGE pattern was obtained when analyzing <i>Campylobacter</i> isolated from flies and broilers.	(Hald et al., 2004)
Beetles	<i>flaA</i> -SVR sequencing pointed that the same <i>Campylobacter</i> type was detected on beetles and in broilers.	(Bates et al., 2004)
Wild birds	The same FAFLP type was detected in faeces of wild birds and broilers.	Messens et al. (2009)
Wild mammals	Common clonal lines among wildlife and chicken isolates were identified within serotype O:2 and the O:4 complex using <i>flaA</i> -RFLP and macrorestriction profiles +	(Petersen et al., 2001a)
<u>Humans</u>		
Depopulation event	The same PFGE pattern was obtained in thinned broiler flock and on catchers, their clothing and equipment, and the modules, crates, and trailers used for transportation.	(Allen et al., 2008)
Boots	The same PFGE pattern was found on farmer boots and in broiler flock.	Messens et al. (2009)
<u>Water</u>		
Drinking line	PFGE patterns of isolates from the drinking line were similar to isolates from subsequent flocks.	(Cokal et al., 2011)
Header tank	Sequence type present in the header tank was similar to the one isolated from broiler flock.	(Ogden et al., 2007)
<u>Environmental sources</u>		
	The same <i>Campylobacter</i> type was isolated from environmental samples (air, soil, etc.) and colonized broiler flock.	(O'Mahony et al., 2011)
Water puddle	The same <i>Campylobacter</i> type distinguished by FAFLP and PFGE was isolated from water puddles and colonized broiler flock.	(Messens et al., 2009)

Multiple studies have highlighted the impact of personnel on *Campylobacter* flock status by detecting *Campylobacter* on their boots, cloths and hands. While farmer might be a vector for *Campylobacter* transmission between houses within a farm, catching crew, truck drivers or the other personnel can be involved in the between farms transmission. The same *Campylobacter* genotype that colonized broiler chickens, was found frequently on personnel boots (Messens et al., 2009). Furthermore, also equipment used by farm workers might be contaminated with *Campylobacter* and it has been proven that

*Campylobacter* genotype detected on crates before transport can infect remaining broilers (Allen et al., 2008).

Despite the absence of many classic stress response mechanisms, *Campylobacter* can survive in water in both biofilms (Buswell et al., 1998) and protozoa (Baré et al., 2010). In genotyping studies, the molecular link between isolates from water or water system and broilers has been reported (Cokal et al., 2011; Ogden et al., 2007). Consequently, based on the available data, the role of water as a *Campylobacter* vehicle cannot be ignored. Additionally, it has been suggested by Herman et al. (2003) and Ogden et al. (2007) that when birds are colonized, contaminated drinker surfaces allows extensive cross-contamination and rapid transmission throughout the house.

*Campylobacter* was also detected in a wide range of environmental samples (i.e. air in broiler house, concrete surrounding and driveways, soil, grass, puddle, foot bath, etc.) before chickens were placed in the broiler house or before they become positive (Hansson et al., 2007; O'Mahony et al., 2011). Based on this finding, it can be concluded that the general surrounds is a step in the pathway from a reservoir to the flock, rather than direct reservoir itself.

#### 4.1.4 Factors associated with *Campylobacter* broilers colonization

Multiple factors have been identified to be associated with the increased risk of *Campylobacter* flock colonization. The most often elucidated factor was the season of rearing, with a higher incidence of colonization during the summer and autumn (Allain et al., 2014; Bouwknecht et al., 2004; Chowdhury et al., 2012; Ellis-Iversen et al., 2009; Kapperud et al., 2009; McDowell et al., 2008a; Refrégier-Petton et al., 2001). The seasonality of *Campylobacter* colonization in broilers is not fully understood yet, although the temperature might be indirectly associated with a higher percentage of *Campylobacter* positive broiler flocks during the warmer months (Jacobs-Reitsma et al., 1994). Certainly, during summer broilers are more exposed to the environment due to intensive broiler house ventilation (Newell and Fearnley, 2003). Probably, also the presence of flies and other insects during warmer seasons as well as better survival of *Campylobacter* in the reservoirs contributes to the higher risk of *Campylobacter* colonization (Ellis-Iversen et al., 2009; Hald et al., 2008).

It has been also reported that the risk of *Campylobacter* colonization of broilers is related to the age of chickens (Barrios et al., 2006; Bouwknecht et al., 2004; Chowdhury et al., 2012; McDowell et al., 2008a; Torralbo et al., 2014). Maternal immunity protects young chickens in first weeks of their live but it does not last long enough to cover the entire production period of broiler chickens (Sahin et al., 2003). An extended rearing time could be also related to the increased opportunity for *Campylobacter* to induce into the broiler flock from the environment around the house (Ridley et al., 2011).

The increased risk of *Campylobacter* colonization of broiler chickens has been also associated with the presence of the other animals (including pets) at the farm or in close neighborhood (Hansson et al., 2010). Probably, this reflects the chance of infection being trafficked from *Campylobacter* positive livestock to broiler house. Similarly explained might be the relation between the number of broiler houses and the higher risk of *Campylobacter* positive broiler flocks (Rushton et al., 2009).

Generally, increased biosecurity (*i. e.* hygiene barrier, limited human traffic, rodent control, house specific clothes, age of broiler house, drinking water treatment, type of the ventilation system) has been identified as a protective factor against *Campylobacter* colonization of broiler flocks (Allain et al., 2014). As confirmed by the molecular studies, human and rodent/insect traffic is an important route for introduction of *Campylobacter* from the external environment (Agunos et al., 2014). Therefore, limitation of human traffic and implementation of hygiene measures might result in lower *Campylobacter* prevalence (Chowdhury et al., 2012). Likewise, exclusion of rodents from broiler houses and insects due to control program or good technical condition of broiler house can lead to lower *Campylobacter* colonization rate (McDowell et al., 2008a).

*Campylobacter* can survive in water for weeks and contamination of on-farm water reservoirs may enable the delivery of live *Campylobacter* to poultry houses (EFSA, 2011a). As such, water treatment as an additional biosecurity measure might be related with lower risk of *Campylobacter* colonization (Allain et al., 2014; Torralbo et al., 2014).

Partial depopulation (thinning), which consists of removing part of the flock before the end of the rearing period is mainly performed to allow additional weight gain in the remaining birds according to the council directive 2007/43/EC (Anonymous, 2007). The positive association between the partial depopulation and *Campylobacter* colonization might be a result of biosecurity breakdown due to the extensive use of catching equipment and the entrance of catching crews into the house (Hald et al., 2000; Torralbo et al., 2014). On the other hand, a Dutch study reported a limited impact of partial depopulation on *Campylobacter* prevalence (Russa et al., 2005).

Another potential risk factors is the *Campylobacter* positive status of a previously reared flock in the same broiler house (Chowdhury et al., 2012). Flock to flock carry-over of the *Campylobacter* infection has been related with the efficiency of cleaning and disinfection as well as down-time between flocks (EFSA, 2011a). Nevertheless, molecular as well as longitudinal studies have revealed limited carry-over effect of *Campylobacter* infection between subsequently reared flocks (Alter et al., 2011; McDowell et al., 2008).

## **4.2. Effect of transport on the *Campylobacter* carriage**

Despite the cleaning and disinfection procedures, transporting crates could be contaminated with *Campylobacter* and they can contribute to the external contamination of transported negative broilers (Rasschaert et al., 2007b). Nevertheless, this event is of low interest for public health due to limited *Campylobacter* numbers on cleaned and disinfected crates, usually detected after enrichment procedure.

In contrast, when *Campylobacter* positive birds are transported, dissemination of *Campylobacter* contamination is reflected by a significant increase of *Campylobacter* counts in faeces (Whyte et al., 2001), and externally on the skin and feathers of transported birds (Stern et al., 1995).

## **4.3. *Campylobacter* on broiler carcasses.**

### **4.3.1 Prevalence**

As reported by European Food Safety Authority, the EU average prevalence of *Campylobacter*-contaminated broiler carcasses was 75.8 % (EFSA, 2010a). Similarly to the *Campylobacter* prevalence at the farm level, also percentage of carcass contamination differs between EU countries (from 4.9 % in Estonia to 100.0 % in Luxemburg). In the same report it was mentioned that *Campylobacter* was detected on 52.7 % of broiler carcasses slaughtered in Belgium. When analyzing 0.01 g of chicken neck skin the *Campylobacter* prevalence range between 27.0 and 33.9 % in years 2000 – 2003 (Ghafir et al., 2007). Similar results (28.5 %) were obtained when analyzing poultry carcasses and poultry products at the retail level from January 1997 to May 1998 (Uyttendaele et al., 1999).

As described in point 2.1, *Campylobacter* is unable to multiply outside the host and it is subjected to multiple stress factors during food chain. Therefore, initially high numbers of *Campylobacter* need to be present on carcass in order to reach the consumer level. Based on this consideration, *Campylobacter* enumeration is essential in order to have insight into the posed risk to humans.

### **4.3.2 Enumeration**

As it has been reported in multiple risk assessment studies, the main consumer risk is associated with highly contaminated products, rather than *Campylobacter* present as such (Nauta et al., 2009). Additionally, Callicott et al. (2008) indicated that *Campylobacter* genotypes detected in humans are originating from highly contaminated carcasses. Therefore, intervention strategies should be aimed at reduction of *Campylobacter* counts in order to reduce public health risk of campylobacteriosis. According to EFSA, a public health risk reduction of > 50 % or > 90 % could be achieved if all batches would comply

with a limit of 1000 or 500 cfu/g of neck and breast skin, respectively (EFSA, 2011a). Therefore, in the frame of public health and food safety, *Campylobacter* enumeration seems to be more relevant than presence/absence testing and this thesis will further, primarily focus on the *Campylobacter* counts.

Enumeration of *Campylobacter* on breast and neck skin samples during the EFSA baseline survey presented the substantial variability between participating countries. The highest proportion of low contaminated carcasses (< 10 cfu/g) was observed in Norway with 98.7 %, whereas the proportion of highly contaminated carcasses (>10 000 cfu/g) was the biggest in Malta with 31.9 %. On average in EU, 21.6 % of tested carcasses carried more than 1 000 cfu/g of breast/neck skin sample (EFSA, 2010a). As presented by Habib et al. (2012), 20.6 % of tested samples in Belgium within the EU baseline survey were contaminated with more than 1 000 cfu/g.

Additionally, as noted in the study of Habib et al. (2012), there was considerable variation in *Campylobacter* contamination between slaughterhouses. Similarly, in the EFSA report the proportion of carcasses with higher *Campylobacter* counts varied significantly between countries and between slaughterhouses within countries. Based on these considerations, it was suggested that certain slaughterhouses are more capable than others in preventing high *Campylobacter* counts on the carcasses. This implies that slaughterhouse processing offers an opportunity for *Campylobacter* risk mitigation (EFSA, 2010b), although evidence-based interventions are not yet available.

#### 4.3.3. *Campylobacter* enumeration methods

A common method used for the enumeration of *Campylobacter* in foods is ISO 10272–2 (ISO, 2006), which utilizes modified charcoal cefoperazonedeoxycholate agar (mCCDA). The drawback of this medium is that while being selective, it does not provide any differential properties, which leads to extensive and time consuming colony confirmations (Ahmed et al., 2012).

Additionally, *Campylobacter* colonies have tendency to spread on mCCDA plates and together with their grey color on black media makes them difficult to quantify (Ahmed et al., 2012; Habib et al., 2011; Ugarte-Ruiz et al., 2012). Recently it has been reported, that CampyFood agar® (CFA, bioMérieux) showed an attractive performance for easy and precise *Campylobacter* enumeration (Habib et al., 2011; Ugarte-Ruiz et al., 2012). However, Ahmed et al. (2012) have indicated that *Campylobacter* counts recovered by CFA are in comparison to mCCDA plates. Other plating media (*i.e.* *Campylobacter* Selective Agar (CASA) (AES Chemunex) and Brilliance™ CampyCount (BBCA) (Oxoid)) have been identified as a possible alternative for the mCCDA although future work is required to develop selective and differential agars with improved accuracy in the enumeration of *Campylobacter* and the properties to eliminate background microflora (Ahmed et al., 2012).

Enumeration of *Campylobacter* by qPCR has been also reported (Botteldoorn et al., 2008; Melero et al., 2011). The main advantages are faster performance and high specificity. However, when using qPCR also dead cells can be detected providing unreliable results (Josefsen et al., 2010). Recent studies indicated that application of propidium monoazide (PMA) or ethidium monoazide (EMA) treatment before the qPCR reaction can differentiate dead *Campylobacter* cells and consequently improve the enumeration precision (Duarte et al., 2015; Josefsen et al., 2010).

#### *4.3.4. The influence of processing operations on the Campylobacter contamination of broiler carcasses during slaughter of Campylobacter positive batches.*

On average more than seventy percent of broiler flocks slaughtered in EU are colonized by *Campylobacter* (point 4.1). The *Campylobacter* counts found in caeca at the end of the rearing period can range between 1.7 and 8.6 log<sub>10</sub> cfu/g (Hansson et al., 2010). Additionally, before slaughter, broilers can be contaminated externally with more than 7.1 log<sub>10</sub> cfu per chicken (Stern et al., 1995). As such, contamination of carcasses cannot be avoided completely during the slaughter process...

Already at the beginning of the slaughter line carcasses can carry more than 3 log<sub>10</sub> cfu/g of *Campylobacter* externally (Berrang and Dickens, 2000; Elvers et al., 2011; Izat et al., 1988; Wempe et al., 1983). Such a high level of *Campylobacter* can be probably explained by the transportation stress and intensive defecation during transport (Whyte et al., 2001).

It has been reported that *Campylobacter* counts on process carcasses decrease during scalding (Berrang and Dickens, 2000; Izat et al., 1988). The reason for that might be high scalding water temperature (Yang et al., 2001) or simple dilution effect.

Nevertheless, *Campylobacter* carcass contamination increases again during plucking (Berrang and Dickens, 2000; Izat et al., 1988). It has been suggested that the rubber fingers in the mechanical picker act to cross-contaminate birds that previously had low or undetectable levels of *Campylobacter* (Keener et al., 2004). Additionally, Berrang et al. (2001) have proven that an increase in the recovery of *Campylobacter* after plucking can be related to the escape of contaminated feces from the cloaca during plucking. *Campylobacter* was also detected in aerosols in plucking rooms (Allen et al., 2007), although airborne contamination does not contribute to the high numbers of *Campylobacter* routinely found on carcasses after plucking (Berrang et al., 2004).

During the evisceration process rupture of the intestines might be observed, causing spillage of intestinal content on carcasses and slaughterhouse environment. Different trends in *Campylobacter* counts have been reported after evisceration. Rosenquist et al. (2006) have noted significant increase in *Campylobacter* counts after evisceration when comparing to after plucking in one of the investigated slaughterhouses. However, in the second one this trend was not present. Likewise, Berrang and Dickens



(2000) and Reich et al. (2008) did not observe changes in *Campylobacter* counts during evisceration process. On the other hand, Allen et al. (2007) have reported that for most flocks, *Campylobacter* numbers per carcass were lower after evisceration than after plucking.

Even though *Campylobacter* can be recovered from the crop (Rasschaert et al., 2007a), there is no knowledge about the quantitative effect of the crop puller on *Campylobacter* carcass contamination. Similarly limited studies have been conducted on evaluating the performance and effectiveness of poultry washers. Bashor et al. (2004) observed that a single inside-outside washer was effective in reducing *Campylobacter* counts on average by 0.31 log<sub>10</sub> cfu. Based on the swab samples collected in three broiler slaughterhouses, *Campylobacter* counts were decreased during washing in two companies, although the effect of washing was not seen in the third slaughterhouse (Izat et al., 1988).

In general, chilling is expected to reduce *Campylobacter* counts on broiler carcasses. In USA and the other countries, where addition of chemical compounds such as chlorine, ozone and chlorine dioxide is allowed, water chilling is commonly used. As expected, significant reduction in *Campylobacter* counts has been reported when water chilling with addition of chemicals is applied (Berrang and Dickens, 2000). In contrast, in the EU, where no chemical substances are authorized for decontamination of poultry carcasses, the air chilling is used more often by broiler slaughterhouses. According to the results of laboratory experiment conducted by Berrang et al. (2008), *Campylobacter* counts were approximately 0.5 log higher on carcasses subjected to air chilling as compared with those subjected to the ice-water immersion-chilling technique. The authors suggested that such a difference might be caused by the washing-off effect in the chilling tank. Likewise, it has been reported that air- and immersion-chilled carcasses without chemical intervention are microbiologically comparable (Huezo et al., 2007). Under such processing conditions no significant difference was seen in *Campylobacter* counts on carcasses after chilling between two Danish slaughterhouses: one using air chilling and the second one spin chiller (Rosenquist et al., 2006).

Multiple studies mentioned in this introduction have described quantitative *Campylobacter* carcass contamination during broiler slaughter. However, in most case authors focused on selected process operations (e.g. plucking, evisceration and chilling) and did not examine the influence of other steps of the slaughter process possibly influencing *Campylobacter* counts on carcasses such as crop puller or washing. However, even more importantly, little is known about *Campylobacter* inter- and intra-slaughterhouse variability and it is not clear what causes the differences between the slaughterhouses.

#### 4.3.5. Contamination of carcasses originating from *Campylobacter* negative batches

Genotyping studies have provided evidence for cross-contamination of carcasses from *Campylobacter* negative batches (Miwa et al., 2003; Rasschaert et al., 2006). However, according to Johannessen et al.

(2007) and Nauta et al. (2005) the cross-contamination occurs shortly and only the first carcasses from a negative batch slaughtered after a positive one become contaminated with *Campylobacter*. In contrast, in a British study (Elvers et al., 2011) it has been suggested that carcasses from a *Campylobacter* negative batch processed immediately after a *Campylobacter*-positive batch can be contaminated at a similar level to those from a positive batch. Therefore, further research might be required to verify the role of cross-contamination in the frame of *Campylobacter* quantification.

## **5. Interventions to control *Campylobacter* in the broiler meat production**

Since broiler meat has been identified as the main source of campylobacteriosis in humans, interventions to control *Campylobacter* in this area seem to be essential in order to protect public health. As presented by the example of Iceland, New Zealand and Denmark, incidence rate of campylobacteriosis can be decreased by the collective contribution of synchronous implemented interventions at all stages of the production chain, *i.e.* during primary production, transportation slaughter, dressing, meat preparation and consumption (Rosenquist et al., 2009; Sears et al., 2011; Tustin et al., 2011).

### **5.1. Interventions against *Campylobacter* at the farm level**

Preventing *Campylobacter* from entering the flock, would have probably the highest impact on the reduction of public health risk. It would not only eliminate the contamination of broiler meat, but also the environmental source of *Campylobacter* exposure to humans (Wagenaar et al., 2013).

#### **5.1.1 Biosecurity measures**

Biosecurity is a set of preventative measures implemented to reduce the risk of transmission of infectious disease from reservoirs of the infectious agent to the target host (EFSA, 2011a).

Even though in practice it is difficult to retain *Campylobacter* free broiler status until the end of the rearing period, the role of biosecurity should not be underestimated. Strengthened biosecurity measures can also significantly delay the introduction of *Campylobacter* into the broiler flock what consequently will reduce the proportion of positive birds entering the slaughterhouse (Gibbens et al., 2001). Processing of the partially colonized flock may result in lower *Campylobacter* counts on produced carcasses in comparison to flocks with high within flock prevalence.

Based on the field trials, it was observed that application of hygienic measures, such as hand wash and sanitizer, separate boots, tools and protective clothing for each broiler house, disinfection footbath, the control of rodents and insects, broiler house maintenance cleaning and disinfection, can prevent broilers from the *Campylobacter* colonization (Gibbens et al., 2001; van de Giessen et al., 1998).

### 5.1.2 Fly screens

As mentioned in point 4.1.3, flies were identified as a potential *Campylobacter* transmission vector and their inflow into the broiler house can range between 2 and 180 thousands per rearing period (Hald et al., 2008). It has been shown in Denmark that installation of fly screens limiting the flying insects' traffic can significantly reduce the prevalence of *Campylobacter* positive flocks from 51.4 in control broiler houses to 15.4 in case control houses during the summer 2006 (Hald et al., 2007). Also, based on the long term observation (4 years) the effectiveness of fly screens was confirmed. Percentage of positive flocks in 10 fly-screened broiler houses was equal to 10.3 in years 2006-2009 in comparison to 41.4% in years 2003-2005 (Bahrndorff et al., 2013) when fly screens were not installed.

### 5.1.3 Drinking water treatment and water additives

Drinking water is also related to the biosecurity at the farm level. As mentioned previously (point 4.1.3), *Campylobacter* was detected in water supply systems of broiler houses, being a possible source of broiler infection.

In Europe broiler farms are mostly supplied with potable water and even if it is chlorinated, the level of sanitizer used is too low to decrease the prevalence of colonization by *Campylobacter* (Stern et al., 2002).

Although water treatment (e.g. by chlorination, filtration or UV irradiation, application of organic acids) has a meaningless effect on the *Campylobacter* reduction in broilers' caeca (Chaveerach et al., 2004; Hermans et al., 2012; Hilmarsson et al., 2006; Metcalf et al., 2011), it could prevent or diminish *Campylobacter* transmission via the water supply systems at the broiler farms. Therefore, it is recommended to implement this intervention (EFSA, 2011a) to successfully eliminate *Campylobacter* from water (Chaveerach et al., 2002; Thormar et al., 2006) and consequently to slow down *Campylobacter* spread within a flock.

### 5.1.4 Feed additives

In this paragraph we refer to chemicals (e.g. organic acids) as feed additives. Next to their application in drinking water, organic acids might also be used as feed additives to reduce *Campylobacter* prevalence in poultry. However, *in vivo* trials demonstrated only a limited effect of feed acidification on *Campylobacter* prevalence in broiler flocks and obtained until now contradicting results putting the use of feed additives in question (Hermans et al. 2011).

Solis de los Santos et al. (2009) showed that application of caprylic acid leads to reduction of *Campylobacter* caecal colonization, Additionally, van Gerwe et al. (2010) reported that the addition of a

medium-chain fatty acid mixture to the feed at 1% reduces the probability of broilers becoming colonized. In contrast, addition of butyrate or formic acid had non or low effect on *Campylobacter* prevention (Skanseng et al., 2010; Van Deun et al., 2008) in the same study of Skanseng et al. (2010) indicated that feed with a combination of 1.5 % formic acid and 0.1 % sorbate reduced the colonization of *Campylobacter* significantly, while a concentration of 2.0 % formic acid in combination with 0.1 % sorbate prevented *Campylobacter* colonization in chickens.

As reported by (Hermans et al., 2010), the mucus layer can have protective effect on *Campylobacter* against supplemented medium-chain fatty acids, and as such application of feed additives is up to now practically not achievable as a control measure for *Campylobacter* in broilers. .

#### 5.1.5 Vaccination

A successful chicken vaccine should prevent colonization or cause a strong (more than 2–3 log) reduction in *Campylobacter* caecal colonization level (de Zoete et al., 2007). As reviewed by de Zoete et al. (2007), currently existing vaccination strategies can be divided into following groups (i) protective role of *Campylobacter* specific antibodies, (ii) killed whole cell vaccines, (iii) flagellin-based vaccines and the others.

Already in early nineties, Stern et al. (1990) presented that *Campylobacter* antibodies induced by vaccination in chickens can have protective properties. Later, several vaccination studies aiming to reduce the susceptibility of broiler chickens for *Campylobacter* colonization have been reported, although with different results (Cawthraw et al., 1994; Myszewski and Stern, 1990; Ziprin et al., 2002). It has been suggested that a much stronger immune response has to be generated than is observed during natural colonization of chickens with *Campylobacter* in order to obtain protective response by vaccination (de Zoete et al., 2007).

Killed whole-cell vaccines are relatively safe, cost-effective and easy to produce. However, de Zoete et al. (2007) suggested that the killed *C. jejuni* themselves may not offer strong immune-stimulatory properties or even possess immune-suppressive activities that limit the development of an effective immune response.

Since *Campylobacter* flagellin is crucial for efficient colonization of the chicken gut, multiple studies tested flagellin as potential candidate for protective antigen. Nevertheless, flagellin is modified by glycosylation and undergoes both phase and antigenic variation, which limits the application of flagellin based vaccines (Lin, 2009).

At the moment, the most promising solution is related to the ABC (ATP-binding cassette) transporter protein CjaA. Encouraging results have been reported by the Polish group showing that chickens orally immunized with an avirulent recombinant *Salmonella* strain carrying the *Campylobacter cjaA* gene,

encoding a highly immunogenic lipoprotein developed serum IgY and mucosal IgA antibody responses against *Campylobacter* and were protected against caecal colonization with heterologous wild type *C. jejuni* strain (Wyszyńska et al., 2004). More recent data, confirmed the potential of CjaA- based vaccines for control of *C. jejuni* in poultry (Buckley et al., 2010; Clark et al., 2012).

Unfortunately, despite all the effort, there is no commercially available vaccine to control *Campylobacter* infections in poultry.

#### 5.1.6 Application of bacteriophages

Bacteriophages (often abbreviated to phages) are naturally occurring predators of bacteria that are ubiquitous in the environment. Their specificity against a particular bacterial species and their lack of impact upon other flora make them attractive antibacterial agents (El-Shibiny et al., 2009). Under experimental conditions the use of phages appears promising in reducing *Campylobacter* numbers (Hammerl et al., 2014; Kittler et al., 2013a), although several aspects of phages application raise doubts. First of all, phages are highly specific for a certain host. Very few phages are able to infect different species, and the host range of most of them includes just a number of strains of one bacterial species (Loc Carrillo et al., 2007). Additionally, the extent and duration of *Campylobacter* counts reduction in caeca after dosing is highly variable (El-Shibiny et al., 2009; Wagenaar et al., 2005). Also the development of resistant strains, and their distribution into the farm environment could be complicating factors while continuous monitoring of phage profiles and susceptibility of those *Campylobacter* strains colonizing the flocks would be necessary (Hagens and Loessner, 2010). And last but not least, currently there is no specific EU-regulation regarding the use of phages in primary production (EFSA, 2011a).

#### 5.1.7 Bacteriocins

Bacteriocins are designated as the antimicrobial peptides (AMPs) produced by bacteria with narrow or broad host ranges. Bacteriocins are produced by almost every bacterial species examined to date for the apparent purpose of destroying their competitors. Therefore, bacteriocin-producing bacteria may achieve competitive advantage and function as an innate barrier against pathogens in the gut (Lin, 2009). Currently six purified bacteriocins that reduce *Campylobacter* colonization in broilers have been described (Table 1.3). It has been reported that *Campylobacter* colonization level can be significantly reduced. However, field validation of the *in-vivo* bacteriocin trials is needed before the practical implementation.

**Table 1.3 Bacteriocins that reduce *Campylobacter* colonization in broilers**

Bacteriocin-producing bacteria					
Name	Species	Bacteriocin name	Reduction (log <sub>10</sub> cfu/g)	Treatment time (days)	References
NRRL B-30509	<i>Paenibacillus polymyxa</i>	SRCAM 602	4.6 - 6.3	10	Stern et al. (2005)
NRRL B-30514	<i>Lactobacillus salivarius</i>	OR-7	> 6	10	Stern et al. (2006)
NRRL B-30745	<i>Enterococcus durans/faecium/hirae</i>	E-760	>6.6	10	Line et al. (2008)
NRRL B-30746	<i>Enterococcus faecium</i>	E 50–52	>6.4	15	Svetoch et al. (2008)
NRRL B-50053	<i>Lactobacillus salivarius</i>	L-1077	> 4	40 - 43	Svetoch et al. (2011)

### 5.1.8 Competitive exclusion

This form of treatment is based on application of beneficial micro-organisms derived typically from the gastrointestinal flora of an adult of the species to be treated. Single organism treatments did not result in *Campylobacter* exclusion, although undefined bacterial mixtures have been demonstrated to effectively control *Campylobacter* infections (Stern et al., 2001). The other published studies evaluating defined microbiota have shown variable results (Line et al., 1998; Morishita et al., 1997) although as yet, there is no commercial product that claims good results against *Campylobacter*.

### 5.1.9 Passive immunization

The method lies in the protection of young chicks against *Campylobacter* colonization by administering antibodies. It is believed that the transfer of immunoglobulin (Ig) Y, the major Ig class in chickens, from hen to embryo via egg yolk plays a key role in the immature immune system (Chalghoumi et al., 2009). Contradicting findings have been recently published. Hermans et al. (2014) have shown that *C. jejuni* counts can be reduced by feeding with broilers IgY-rich yolks from hens immunized with *C. jejuni*. In contrast, the study conducted in U.S. has shown no differences in the caecal colonization of *C. jejuni* between hyperimmunized egg yolk powder treated and non-treated control chickens (Paul et al., 2014).

#### 5.1.10 Reduction of slaughter age and discontinued thinning

Based on the risk identification studies (see point 4.1.4) reduction of slaughter age and discontinued thinning have been proposed by EFSA (2011a) as a potential intervention measure at farm level. Nowadays in Belgium, broiler flocks are thinned at the age of 5 weeks and cleared at the age of 6 weeks. Due to the economic reasons implementation of these measures seem to be not achievable.

### 5.2. *Campylobacter* control at the slaughterhouse level

Although mentioned above strengthened biosecurity measures can reduce the proportion of *Campylobacter* positive flocks, sustaining constantly tight hygiene barriers is impossible. Moreover, up to date, all other potential intervention measures (i.e. vaccination, competitive exclusion, bacteriophages, etc.) are under development and they cannot provide reliable solution for rearing *Campylobacter* negative broilers (Hermans et al., 2011; Newell et al., 2011). Based on these considerations, it is highly possible that broilers delivered to slaughterhouse will carry *Campylobacter* in their caeca. As indicated in point 4.3.4, when a *Campylobacter* positive batch is processed, production of *Campylobacter* negative carcasses is practically not feasible. Nevertheless, application of intervention measures can reduce *Campylobacter* numbers on processed poultry meat.

#### 5.2.1 Hygiene measures

Based on (EC) 852/2004 regulation (Anonymous, 2004b), all food chain actors are obliged to implement GHP (Good Hygiene Practices) and HACCP (Hazard Analysis and Critical Control Points) principles in order to improve their food safety output. As reviewed by (Wilhelm et al., 2011), microbial testing post-HACCP implementation resulted in the decrease of aerobic counts and *Salmonella* in comparison to pre-HACCP implementation situation. Furthermore, (Stern and Robach, 2003) speculated that implementation of HACCP regulation requiring lower *Salmonella* prevalence could have also an effect on *Campylobacter* reduction on poultry products.

Improved hygiene control based on the use of water sprays, increased chlorine concentration in water and limited contact between carcasses reduced *Campylobacter* counts on broiler carcasses (Mead et al., 1995). Moreover, Habib et al. (2012) found a possible relationship between operational hygiene inspection scores and *Campylobacter* contamination on broiler carcasses. The bottleneck of the broiler slaughter, in the frame of hygiene, is the evisceration process. Birds' intestines are likely to be ruptured during the evisceration process due to the lack of correct adaptation to the carcass weight or due to the natural variability in the chicken size. During the rupture of intestines, faecal material is released and it can contaminate eviscerated carcasses. In a French study, it has been reported that carcasses with dirty

faecal marks on eviscerated carcasses are 2 times more likely to be contaminated with *Campylobacter* (Hue et al., 2010). According to Sampers et al. (2010), poor evisceration process, in one of the visited companies, resulted in increased numbers and prevalence of *Campylobacter* on poultry meat. Additionally, it has been reported that carcasses with faecal contamination can carry up to 0.9 log<sub>10</sub> cfu *Campylobacter* more on their skin in comparison to carcasses without the visual contamination (Berrang et al., 2004; Boysen and Rosenquist, 2009). In contrast, the differences in the hygiene management between two Brazilian slaughterhouses did not affect the frequencies of *Salmonella* detection (Matias et al., 2010). This findings are in line with the study of Hutchison et al. (2006) who were unable to find meaningful correlations between slaughterhouse hygiene and counts of presumptive indicator bacteria on carcasses. As he further suggested, carcass contamination might be influenced by other external factors possibly associated with the technical aspects of the slaughter process.

### 5.2.2. Decontamination

As contamination of carcasses with *Campylobacter* cannot be avoided during the slaughter process of *Campylobacter* positive batches, physical treatment or application of specific chemicals was studied in the frame of *Campylobacter* counts reduction.

#### Physical decontamination

Physical decontamination includes freezing, crust-freezing, steam treatment, ultrasound treatment, heat treatment, irradiation, etc. or the combination of mentioned methods.

Various studies reported significant reduction in *Campylobacter* counts in poultry meat during freezing. While the greatest decrease of *Campylobacter* counts (*ca.* 1 log<sub>10</sub> cfu) was observed during the first day of storage at -20 to -22 °C, prolonged freezing can further but slower and gradually reduce *Campylobacter* numbers (Bhaduri and Cottrell, 2004; Georgsson et al., 2006; Sampers et al., 2010). To reduce human exposure to the *Campylobacter*, freezing of carcasses originating from *Campylobacter* positive flocks was applied in the frame of national *Campylobacter* control programs in Norway, Denmark and Iceland (Hofshagen and Kruse, 2005; Rosenquist et al., 2009; Tustin et al., 2011). Additionally, a British study (Harrison et al., 2013) suggested that freezing of chicken livers can be a part of routine process decreasing *Campylobacter* numbers on this product. In line with this finding, Sampers et al. (2008) identified freezing as a protective factor in poultry meat preparation companies in Belgium. Crust-freezing, on the other hand, is a method during which the surface of the carcass is temporarily frozen on-line during processing. As estimated by Havelaar et al. (2007), the risk of consumer exposure might be decreased even by 83 % if the crust-freezing is applied. Experimental data presented reduction by approximately 0.5 log<sub>10</sub> cfu on naturally contaminated chicken fillets (Boysen and Rosenquist, 2009)



and inoculated drumsticks (Haughton et al., 2012). To improve the effectiveness in *Campylobacter* counts reduction, crust-freezing can also be potentially use in the combination with steam treatment (James et al., 2007), although no synergism could be observed between crust-freezing and UV treatment (Haughton et al., 2012).

When ultraviolet light was applied at a dose of 0.192 J/cm<sup>2</sup>, reduction by approximately 0.6 - 0.8 log<sub>10</sub> cfu in *Campylobacter* counts was observed in inoculated poultry meat samples (Haughton et al., 2011; Isohanni and Lyhs, 2009). However, increased radiation intensity may contribute to changes in the color of UV-treated poultry meat (Lyon et al., 2007).

The other possibly applied interventions targeting reduction of *Campylobacter* numbers might be steam or hot water treatment (Berrang et al., 2000b; Whyte et al., 2003). It has been also reported that combination of steam/hot water treatment with ultrasound can improve effectiveness of the intervention (Boysen and Rosenquist, 2009; Musavian et al., 2014).

Application of irradiation can be beneficial from the food safety perspective, although consumer opposition to this method based on psychological perception hampers implementation of irradiation in poultry meat production sector (Farkas, 1998).

#### Chemical decontamination

Although (EC) No. 853/2004 regulation (Anonymous, 2004a) foresees chemical decontamination as a supplement to good hygiene practices, at present none of chemical substances is authorized for use in the EU for raw poultry decontamination. Nevertheless, increasing public health risk posed by *Campylobacter* and recent EFSA approval for the use of lactic acid in beef (EFSA, 2011b) open the discussion on the application of chemical treatment in poultry meat production. Organic acids as well as other chemicals have been evaluated for *Campylobacter* reduction in numerous studies (Table 1.4).

As evaluated by EFSA, application of lactic acid treatment at concentration 2 – 5 % is efficient for beef decontamination and it complies with the European Union specifications for food additives (EFSA, 2011b). As reported by Rasschaert et al. (2013), even at lower concentration (1.5 %) lactic acid can be effective in *Campylobacter* counts reduction. A similar reduction for 2.5% lactic acid was obtained in the study of Riedel et al. (2009) who also indicated that extending the treatment time to 15 minutes didn't result in any further reduction.

It has also been shown that a 1 -2 log<sub>10</sub> reduction was obtained when the treatment with other organic acids was applied (Table 1.4). Immersion of carcasses in chlorine solution (100 ppm) for 15 minutes can decrease *Campylobacter* counts by up 0.9 log<sub>10</sub>. Other not-organic chemicals seem to be more effective (Table 1.4).

Most studies performed experiments on inoculated samples under the laboratory conditions with sometimes long contact times. Therefore, it is hard to predict if chemical treatment would have similar

effect when applied in the slaughter line. Also more study is required on the possible implementation in the slaughterhouse. Another aspect that limits the implementation of this control measure is a low consumer acceptability (MacRitchie et al., 2014).

**Table 1.4. Effect of selected, studied chemical treatments on *Campylobacter* reduction in raw poultry**

Substance	Conc.	Contact time	Application	Matrix	Reduction (log <sub>10</sub> )	References
Acidified sodium chlorite	500 ppm	-	Spray; 3 ml	Skin	0.95	(Meredith et al., 2013)
	1200 ppm	-	Spray; 3 ml	Skin	1.38	
	0.1 %	15 s	immersion	Skin	1.6 - 1.9	(özdemir et al., 2006)
	40 ppm	120 s	immersion	Skin	0.4	(Chantarapanont et al., 2004)
	100 ppm	900 s	immersion	Skin	1.1	
	1100 ppm	15 s	Spray; 147 ml	Carcass	2.6	(Kemp et al., 2001)
Acetic acid	0.1 %	60 s	immersion	Wing rinse	1.4	(Zhao and Doyle, 2006)
Capric acid	5 %	60 s	Immersion	Skin	1.78	(Riedel et al., 2009)
Caprylic acid	5 %	60 s	Immersion	Skin	1.35	(Riedel et al., 2009)
Cetylpyridinium chloride	0.35 %	-	spray	Meat	0.8	(Chen et al., 2014)
	0.6 %	-	spray	Meat	0.8	
	0.5 %	60 s	Immersion	Skin	> 4.2	(Riedel et al., 2009)
Chlorine	50 ppm	15 s	Immersion	Carcass	0.4	(Li et al., 2002)
	100 ppm	900 s	immersion	Skin	0.9	(Chantarapanont et al., 2004)
Citric acid	5 %	-	Spray; 3 ml	Skin	1.82	(Meredith et al., 2013)
Formic acid	2 %	60 s	Immersion	Skin	1.57	(Riedel et al., 2009)
Lactic acid	5 %	-	Spray; 3 ml	Skin	1.26	(Meredith et al., 2013)
	1.5 %	180 s	Immersion	Carcass	1.24 - 1.62	(Rasschaert et al., 2013)
	5 %	60 s	Immersion	Skin	1.6	(Chaine et al., 2013)
	2.5 %	60 s	Immersion	Skin	1.69	(Riedel et al., 2009)
	0.07 %	23 s	Immersion	Meat	1.3	(Chen et al., 2014)
Paracetic acid	0.1 %	23 s	Immersion	Meat	1.3	
	0.04 %	20 s	Immersion	Carcass	1.93	(Nagel et al., 2013)
	0.1 %	20 s	Immersion	Carcass	2.03	
	100 ppm	900 s	immersion	Skin	1.0	(Chantarapanont et al., 2004)
	10 %	-	Spray; 3 ml	Skin	1.33	(Meredith et al., 2013)
Trisodium phosphate	14 %	-	Spray; 3 ml	Skin	1.56	
	10 %	60 s	Immersion	Skin	1.74	(Riedel et al., 2009)

### 5.2.3 Logistic and scheduled slaughter

Scheduled slaughter means identifying flocks positive for *Campylobacter* before they are slaughtered and subjecting carcasses from these flocks to special treatment (like freezing) or other *Campylobacter* reducing measures. Whereas logistic slaughter refers to slaughtering positive flocks after negative flocks

to avoid cross-contamination from the positive to negative flocks. Both methods required sampling of broiler batches before slaughter. Since *Campylobacter* can spread rapidly within a flock (van Gerwe et al., 2009), broiler batches should be sampled shortly before the slaughter. This cause not only logistic difficulties but also methodological ones. Method currently applied ISO 10272–1 (ISO, 2006) for *Campylobacter* detection at the farm level is time consuming. Real-time PCR based techniques are not affordable for the clients of many routine laboratories. Therefore, development of the rapid and easy to perform method for *Campylobacter* detection seems to be necessary to consider scheduled or logistic slaughter as potential interventions.

## 6. Closing remarks

*Campylobacter* is more than 30 years after having emerged as an established zoonosis and still it is a challenging food safety problem to tackle. Consumers can protect themselves from campylobacteriosis through careful storage and preparation and proper cooking of poultry meat, In particular the prevention of cross contamination of other foods in the kitchen is very important. However, efforts to reduce the prevalence of *Campylobacter* in the broiler production sector, and its transmission through the supply chain are main priority and responsibility of the broiler production sector and poultry meat processing industry. The above mentioned literature review showed that developing appropriate control measure is still challenging. *Campylobacter* is known to be widespread in the broiler production sector, also in Belgium. The present research focused on methods for quick identification of *Campylobacter* positive flocks and broiler carcasses with high contamination levels and the identification of risk factors that contribute to broiler meat with elevated levels of *Campylobacter* in order to bring guidelines to poultry slaughterhouses on both technical characteristics, operation to control the food safety issue of *Campylobacter* in poultry.

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**AIMS**

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*Campylobacter* continues to be the leading bacterial cause of food-borne gastroenteritis in humans. In Belgium campylobacteriosis is the most frequently reported zoonosis since 2005. Although multiple potential sources of human campylobacteriosis have been identified, approximately 20 to 30 % of cases are related to the handling and consumption of broiler meat. This observation points out that more effort is required to intervene in the broiler meat production chain in order to control *Campylobacter*. The most effective intervention should aim at the reduction of *Campylobacter* prevalence or colonization level at the primary production. Since, there are still no reliable solutions for *Campylobacter* prevention at the farm level available up till now, placing interventions further in the poultry meat supply chain seems more practical achievable in the short term perspective. However, to set up such intervention measures, a good insight in the current situation on *Campylobacter* contamination at the slaughterhouse level is required.

Therefore, **the general aim of this thesis was to achieve a better understanding of the *Campylobacter* contamination during broiler slaughter.**

We attempted to achieve this aim by fulfilling the following objectives:

1. Evaluation of sampling strategies and analytical methodologies for the reliable enumeration of *Campylobacter* colonization level in pre-slaughtered broilers and *Campylobacter* contamination on broiler carcasses during slaughter (**Chapter 1 and Chapter 2**).
2. Investigation of the impact of the slaughter process on *Campylobacter* carcass contamination
  - 2.1 Quantification of the carcass contamination along the slaughter line during the processing of *Campylobacter* positive batches (**Chapter 3**).
  - 2.2 Generating data on *Campylobacter* counts during the consecutive slaughter of broiler batches with a different *Campylobacter* status (**Chapter 4**).
  - 2.3 Identification of factors associated with the level of *Campylobacter* carcass contamination at different steps of the slaughter process. (**Chapter 5**)



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**EVALUATION OF A NEW MEDIUM FOR DIRECT  
ENUMERATION OF *CAMPYLOBACTER* IN POULTRY MEAT  
SAMPLES**

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*Seliwiorstow, T., J. Baré, I. B. Verhaegen, M. Uyttendaele, L. De Zutter (2014): Evaluation of a New Chromogenic Medium for Direct Enumeration of Campylobacter in Poultry Meat Samples. J. Food Protect. 77, 2111-2114.*

## Abstract

The present study was conducted to compare *Campylobacter* counts obtained by three selective media: modified charcoal cefoperazonedeoxycholate agar (mCCDA), Campy Food agar (CFA; bioMérieux, France) and a novel agar: RAPID'*Campylobacter* agar (RAPID; Bio-Rad, France). Analysis of 12 artificially and 36 naturally contaminated samples indicated no significant differences in *Campylobacter* counts obtained by all three selective media. Lin's concordance correlation coefficient (CCC) and Bland–Altman plot revealed a high level of agreement, between *Campylobacter* counts when evaluating RAPID versus mCCDA and CFA plates. RAPID agar was the only medium tested that could effectively suppress the growth of the background microflora with naturally contaminated samples.

Results of this study clearly indicated that RAPID agar is highly selective media without loss of sensitivity for recovering *Campylobacter*. Results obtained are in agreement with other commonly used media, RAPID media is suitable for *Campylobacter* enumeration in poultry meat samples.

## 1. Introduction

Even though number of confirmed reported human campylobacteriosis cases decreased in European Union in 2012, *Campylobacter* still remains the most frequently reported cause of bacterial gastroenteritis (EFSA, 2014). Chicken broiler meat is the main vehicle for campylobacteriosis in humans and accounts for approximately 30 % of all cases in Europe (EFSA, 2011a; Greig and Ravel, 2009). Slaughter and further processing of broiler carcasses carrying *Campylobacter* leads to contamination of broiler meat along the food supply chain (Lienau et al., 2007; Melero et al., 2012).

A decrease of the prevalence of *Campylobacter* at the primary production stage would significantly reduce the public health risk, although at present eradication of *Campylobacter* infection in broiler flocks at the end of the rearing period is not feasible (Hermans et al., 2011; Newell et al., 2011).

The risk of campylobacteriosis might also be limited by interventions later in the food chain, based on reduction or control of the level of *Campylobacter* carcass contamination. Following the successful intervention in New Zealand (Sears et al., 2011), where the mandatory quantitative target for *Campylobacter* on poultry carcasses was introduced, both international and national authorities have consider implementation of quantitative criteria for *Campylobacter* in fresh poultry meat (EFSA, 2011a; Swart et al., 2013). Nevertheless, such a regulation requires a reliable microbiological method for *Campylobacter* quantification. The most commonly applied method for enumeration of *Campylobacter* is the ISO standard 10272-2 (International Organization for Standardization., 2006), which uses modified charcoal cefoperazonedexoxycholate agar (mCCDA) plates. However, quantification of colorless *Campylobacter* colonies on a black-colored agar is difficult and time consuming. In contrast, working with Campy Food agar (CFA) plates is less laborious and leads to the same results (Habib et al., 2011; Ugarte-Ruiz et al., 2012). Nevertheless, when analyzing naturally contaminated samples, background microflora may grow on CFA as well as on mCCDA plates, which may result in overestimation of *Campylobacter* colonies (Ahmed et al., 2012).

The aim of the present study was to evaluate the newly developed RAPID'*Campylobacter* agar (RAPID'*Campylobacter* agar) for quantification of *Campylobacter* in poultry samples, and to compare obtained results with those from widely used mCCDA and CFA plates.

## 2. Materials and methods

### 2.1. Samples overview

Both artificially inoculated (n = 12) and naturally contaminated (n = 36) poultry samples were used within this study.

The artificially inoculated samples were spiked by a third party laboratory. Three of these samples did not contain *Campylobacter* cells, six were contaminated with *Campylobacter jejuni*, and three were contaminated with *Campylobacter coli*, both strains of poultry origin. Inoculum levels were  $10^3$  to  $10^5$  cfu/g. All samples were analyzed on the day of delivery.

Naturally contaminated samples were collected during six visits in broiler slaughterhouses when *Campylobacter* positive batch was slaughtered. The *Campylobacter*-positive status of these birds was established previously at the farm level by examination of caecal droppings for *Campylobacter*. During every slaughterhouse visit, six carcasses were collected aseptically, transported to the laboratory under chilled conditions and analyzed the same day.

### 2.2. Sample preparation and enumeration of *Campylobacter*

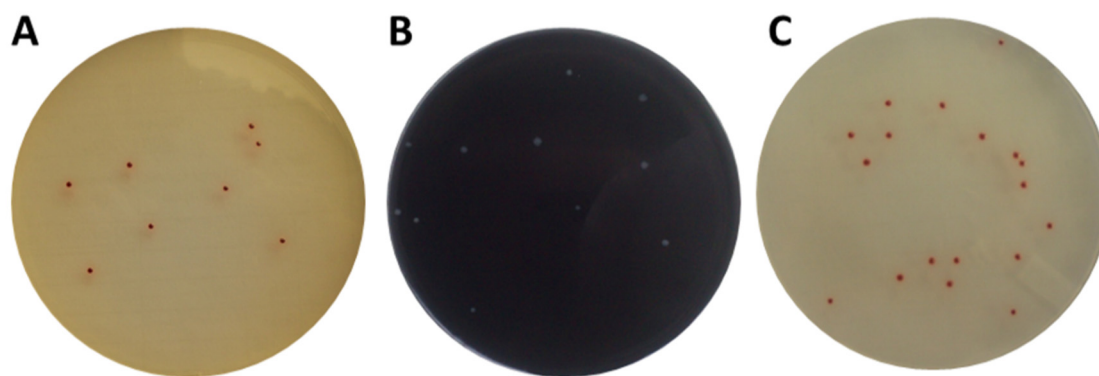
Artificially contaminated samples (10 g) were delivered in chilled conditions in closed plastic bags. For naturally contaminated samples, approximately 10 grams of breast skin was excised from each collected carcass (Baré et al., 2013). All samples (artificially and naturally contaminated) were diluted with 0.1% peptone water (Bio-Rad, Hercules, CA) at a ratio of 1 to 10 and homogenized for 1 minute. Samples were plated in parallel on RAPID (Bio-Rad, Marnes-laCoquette, France), mCCDA (Oxoid, Basingstoke, England) and CFA (bioMérieux, Marcy l'Etoile, France) plates. One ml from the homogenate was spread plated on 2 agar plates (0.5 ml on each plate) and 0.1 ml of further dilutions was plated using the spiral platter (Eddy Jet Spiral Plater, IUL instruments, Barcelona, Spain). After incubation under microaerobic conditions (6 % CO<sub>2</sub>, 6 % H<sub>2</sub>, 4 % O<sub>2</sub>, 84% N<sub>2</sub>) at 41.5 °C for 48 h, colonies with typical *Campylobacter* morphology for each type of agar plate were counted. At least four presumptive positive colonies from every plate type per sample were confirmed by microscopic observation and *Campylobacter* species-level PCR assay to differentiate between *C. jejuni* and *C. coli* (Vandamme et al., 1997).

### 2.3. Data analysis

*Campylobacter* colonies were uncountable on mCCDA plates (because of swarming) for four samples. For one of these samples and two others, *Campylobacter* colonies also spread on the RAPID plates. These six samples and the three *Campylobacter*-negative samples (samples with results below the limit of enumeration of 10 CFU/g) were excluded from analysis. *Campylobacter* counts were  $\log_{10}$ -transformed and differences in results among the three plate types were examined using a repeated measures general linear model with species as a between-subjects variable. A significance level of 5% was used. The agreement between *Campylobacter* counts obtained by different plates was calculated using Lin's concordance correlation coefficient (CCC) (Lin, 1989) which indicates how well *Campylobacter* counts on mCCDA and CFA matches to those on RAPID plates. These calculations were carried out using SAS version 9.4 (SAS Institute, Cary, NC). Additional comparison of *Campylobacter* counts on different agars was conducted by constructing a Bland–Altman plot in SPSS version 22 (IBM, Armonk, NY) (Bland, J Martin. Altman, 1995).

### 3. Results

*Campylobacter* was detected on 9 out of 12 artificially inoculated samples and on all 36 of the naturally contaminated breast skin samples collected after chilling (Table 2.1). Figure 2.1. shows *Campylobacter* colonies on RAPID (Fig. 1A), mCCDA (Fig. 1B), and CFA (Fig. 1C) plates.



**Figure 2.1. Morphology of *Campylobacter* colonies recovered from naturally contaminated chicken broiler skin samples on RAPID (A), mCCDA (B), and CFA (C) plates**

As expected, in samples which were not inoculated ( $n=3$ ) had no *Campylobacter* growth on mCCDA as well as on the CFA and RAPID plates. On mCCDA and RAPID plates *Campylobacter* was not quantified for 4 and 3 of the naturally contaminated because of *Campylobacter* swarming. In one of these samples,

*Campylobacter* colonies were swarmed on both mCCDA and RAPID plates; thus 6 samples being excluded from the analysis.

**Table 2.1. Comparison of *Campylobacter* counts on different media for artificially and naturally contaminated samples.**

Plate	Negative	Uncountable <sup>a</sup>	<i>Campylobacter</i> counts (log <sub>10</sub> cfu/g)		
			Mean ± SD	Minimum	Maximum
<i>Artificially inoculated samples</i> (n=12)					
mCCDA	3	0	4.16 ± 1.07	2.69	5.45
CFA	3	0	4.10 ± 0.85	2.92	5.22
RAPID	3	0	4.11 ± 0.99	2.61	5.23
<i>Naturally contaminated samples</i> (n=36)					
mCCDA	0	4	2.38 ± 0.65	1.00	3.85
CFA	0	0	2.49 ± 0.69	1.48	4.21
RAPID	0	3	2.44 ± 0.77	1.00	4.26

<sup>a</sup> Uncountable samples due to swarming of *Campylobacter* colonies

n – number of tested samples

SD – standard deviation

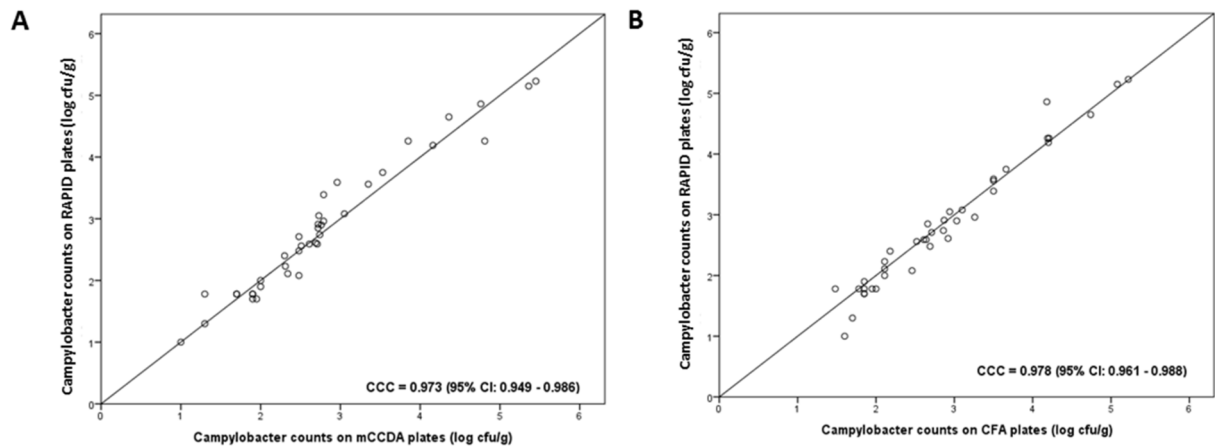
Mean *Campylobacter* counts from artificially contaminated samples were 4.10 to 4.16 log<sub>10</sub> cfu/g and no significant differences between plates were found with repeated measures general linear model ( $P=0.971$ ) (Table 2.1.).

When analyzing naturally contaminated samples, only RAPID agar completely inhibited the growth of the background microflora. With repeated measures general linear model, no significant differences between agars were detected when comparing *Campylobacter* counts from naturally contaminated samples ( $P=0.110$ ) (Table 2.1.).

*C. jejuni* and *C. coli* was recovered in twenty seven and nine naturally contaminated samples, respectively. No significant difference in counts was found between *Campylobacter* species ( $P>0.2$ ). Additionally, differences in *Campylobacter* counts were independent from *Campylobacter* species for any of tested agars ( $P>0.1$ ).

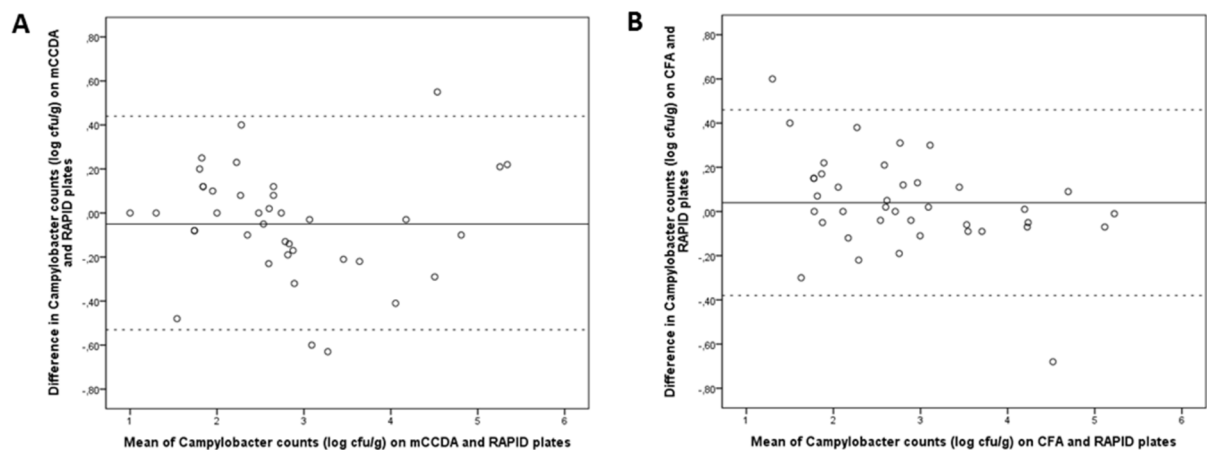
Close agreement between *Campylobacter* counts was found between RAPID and the other two media. The CCC for overall (artificially and naturally contaminated samples) *Campylobacter* counts on RAPID and on mCCDA was 0.973 (95% CI: 0.949 - 0.986) (Figure 2.2A). For CFA versus RAPID, the CCC was 0.978 (95% CI: 0.961 - 0.988) (Figure 2.2B).





**Figure 2.2.** Concordance correlation coefficient (CCC) plot between *Campylobacter* counts obtained on mCCDA and RAPID *Campylobacter* agar (A), and between *Campylobacter* counts obtained on CFA and RAPID *Campylobacter* agar (B). The solid line represents the line of perfect concordance.

the Bland-Altman plot (Figure 2.3) shows little variation in *Campylobacter* counts between RAPID and mCCDA (A) and CFA (B). The main difference between RAPID and CFA plates was 0.04 log<sub>10</sub> cfu/g and we estimated that for 95% of measurements counts on RAPID will be situated between -0.38 log<sub>10</sub> cfu/g below counts on CFA and 0.46 log<sub>10</sub> cfu/g above it. Similar narrow intervals were indicated when plotting averages against differences in *Campylobacter* counts on RAPID and mCCDA plates. The main difference accounted for -0.05 log<sub>10</sub> cfu/g and 95% limits of agreement were -0.53 and 0.44 log<sub>10</sub> cfu/g.



**Figure 2.3.** Bland–Altman plot illustrating the agreement between *Campylobacter* counts obtained on mCCDA and RAPID *Campylobacter* agar (A), and between *Campylobacter* counts obtained on CFA and RAPID *Campylobacter* agar (B). The main difference is indicated by the solid line and 95% limits of agreement by the dashed lines

### 3. Discussion

Implementation of the quantitative criteria for *Campylobacter* in fresh poultry meat requires reliable technic for *Campylobacter* quantification. Development of a highly selective media, suppressing growth of background microflora might limit the number of confirmation test and consequently reveal feasibility for cost effective and simple *Campylobacter* enumeration. The present study It is the first comparative performance of a new chromogenic media RAPID'*Campylobacter* agar (RAPID) against charcoal cefoperazonedeoxycholate agar (mCCDA), recommended by ISO 10272-2, and another commercially available medium Campy Food agar (CFA) for *Campylobacter* enumeration in poultry samples.

No significant differences were detected between *Campylobacter* counts on three agar types for artificially and naturally contaminated samples. Habib et al. (2011) and Ugarte-Ruiz et al.(2012) compared *Campylobacter* counts on mCCDA and CFA but found no significant differences between these two media. However, Ahmed et al. (2012) found were significantly lower *Campylobacter* counts on CFA than those on mCCDA for artificially contaminated samples and that the recovery rate on different media differed with *Campylobacter* species. In contrast, in the present study, no significant difference in *Campylobacter* counts was observed between the three media evaluated. One explanation for this difference is that the tested samples could have contained different *Campylobacter* strains.

Obtained results revealed a high level of agreement between results on RAPID plates and those on mCCDA and CFA plates, as demonstrated by concordance correlation coefficient and the Bland–Altman plot. In agreement with our findings, high concordance in *Campylobacter* counts recovered by CFA and mCCDA plates has been previously reported (7).

In concordance with previous studies (Ahmed et al., 2012; Habib et al., 2011), growth of non-*Campylobacter* colonies was observed on both mCCDA and CFA plates. Therefore, correct identification and quantification of *Campylobacter* on mCCDA and CFA plates requires trained and experienced personnel and time for confirmation tests (i.e. microscopic observation, biochemical testing or PCR). In contrast, background microflora was absent on RAPID plates.

As reported previously (Gharst et al., 2013), in the present study *Campylobacter* colonies swarmed on some mCCDA plates, making it impossible to obtain accurate colony counts. Similar swarming occurred on RAPID medium, but on CFA plates *Campylobacter* colonies from all samples had a regular round shape. This difference between media might be explained by the variability in the humidity of the plates. Although in the present study all plates were dried for the same amount of time (30 min) before inoculation, additional experiments revealed that a longer drying period can inhibit the spread of *Campylobacter* colonies on RAPID plates.

In conclusion, the present study indicates a high level of agreement in *Campylobacter* counts was found for RAPID plates and the other two media (mCCDA and CFA). RAPID agar also was the only media out of

three tested that effectively suppressed the growth of the background microflora in naturally contaminated samples. Consequently, the new chromogenic medium is a reliable alternative for less labor intensive *Campylobacter* quantification from poultry samples.

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**COMPARISON OF SAMPLE TYPES AND ANALYTICAL  
METHODS FOR THE DETECTION OF HIGHLY  
*CAMPYLOBACTER* COLONIZED BROILER FLOCKS AT  
DIFFERENT STAGES IN THE POULTRY MEAT PRODUCTION  
CHAIN.**

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Comparison of Sample Types and Analytical Methods for the Detection of Highly Campylobacter-  
Colonized Broiler Flocks at Different Stages in the Poultry Meat Production Chain. Foodborne Pathog. Dis.  
In press (doi: 10.1089/fpd.2014.1894)*

## Abstract

Exclusion of broiler batches, highly colonized with *Campylobacter* ( $>7.5 \log_{10}$  cfu/g of caecal content), from the fresh poultry meat market will decrease the risk of human campylobacteriosis. The objective of this study was to compare different sample types (both at the farm and the slaughterhouse) and methods (direct culture, qPCR, PMA-qPCR) applied for the quantification of the *Campylobacter* colonization level. In addition, the applicability of the lateral flow based immunoassay, Singlepath® Direct Campy Poultry test (Singlepath® test), was evaluated as a rapid method for the qualitative detection of *Campylobacter* in highly colonized broiler batches.

*Campylobacter* counts differed significantly between sample types collected at farms (caecal droppings, faeces, boot swabs) and at slaughterhouses (caecal content, faecal material from crates). Furthermore, comparison of *Campylobacter* counts obtained by different methods (direct culture, qPCR, PMA-qPCR) in caecal droppings revealed significant differences, although this was not observed for caecal content samples. Evaluation of the Singlepath® test on caecal droppings and caecal content samples revealed an acceptable level of sensitivity and specificity.

In conclusion, caecal droppings and caecal content are proposed as the most representative sample types for quantification of *Campylobacter* colonization level of broilers at farm and slaughterhouse, respectively. Direct culture and qPCR are equally sensitive for quantification of *Campylobacter* in fresh caecal content samples. PMA treatment before qPCR inhibits the signal from dead *Campylobacter* cells. Consequently, when samples are extensively stored and/or transported qPCR is preferred to direct culture and PMA-qPCR. Furthermore, the Singlepath® test offers a convenient alternative method for rapid detection of *Campylobacter* in highly colonized broiler batches.

## 1. Introduction

Campylobacteriosis is the most commonly reported zoonosis, with over 200,000 confirmed and nine million estimated cases per year in the EU (EFSA, 2014, 2011). Although variable sources of human infection have been reported (Taylor et al., 2013), most human campylobacteriosis cases are related to handling and consumption of poultry meat (Greig and Ravel, 2009; Pires et al., 2010).

Contamination of poultry carcasses with *Campylobacter* occurs during slaughtering of *Campylobacter* positive flocks (Sampers et al., 2010). Moreover, based on the risk assessment study (Nauta and Havelaar, 2008), it has been indicated that the main source of infection for consumers is fresh broiler meat originating from batches colonized with *Campylobacter* at levels of 7 to 8 log<sub>10</sub> cfu/g. As such, quantification of the *Campylobacter* colonization level is a more efficient risk indicator than presence-absence testing (Nauta et al., 2009). Nevertheless, the identification of highly colonized *Campylobacter* batches requires representative sampling strategies and also reliable methods for quantification of the *Campylobacter* colonization level of birds before slaughter.

Several sample types, such as caecal droppings (Herman et al., 2003), faecal material (Stern et al., 2003), boot swabs (Chowdhury et al., 2012) at the farm level and caecal content (Rasschaert et al., 2007) and faecal material from crates (Nauta et al., 2009) at the slaughterhouse level have been used to determine the *Campylobacter* status of slaughter birds. However, the type of sample can influence *Campylobacter* counts, providing unreliable information on the flock colonization level. Therefore, at first, a comparative analysis of sample types for *Campylobacter* quantification by direct culture method at farm and slaughterhouse level was performed.

The most commonly used method for quantification of *Campylobacter* colonization level is the direct culture on selective agar although it is a time consuming procedure that is not applicable for testing all broiler flocks shortly before slaughter. A quicker alternative might be quantitative real-time PCR (qPCR) or a qPCR combined with propidium monoazide (PMA), which quantify only viable *Campylobacter* cells (Duarte et al., 2015; Josefsen et al., 2010). Thus, secondly, direct culture, qPCR, and PMA-qPCR were evaluated for their usefulness for *Campylobacter* enumeration on both caecal droppings and caecal content samples.

Nevertheless, culture and molecular methods require either time or advanced laboratory equipment. Therefore, a rapid, simple, and reliable test identifying highly colonized broiler flocks might allow testing of all poultry broiler batches shortly before the slaughter. For this reason, the utility of a recently developed lateral flow test, namely, the Singlepath® Direct Campy Poultry (Singlepath® test), was evaluated.

## 2. Materials and methods

### 2.1. Sample collection

At farms, from each of 10 broiler flocks (i.e. a group of chickens reared in the same broiler house) at the age of 39 to 41 days, at least 10 fresh caecal droppings (i.e. watery and light brown in color; Fig. 3.1) and 10 fresh faecal material samples (i.e. firm and brown droppings with a white part made from urates) were collected and pooled in sample cups with a cover to minimize sample exposure to the air. For each of these flocks, two boot swabs (Art: H01038A, KOLMI, France) were also analysed. At the slaughterhouse level, 10 batches (i.e. birds from one flock slaughtered at the same day), that were not related to previously examined flocks at the farm level, were sampled by collecting 6 intestinal packages in plastic bags. Additionally, for each batch the correspondent faecal material (ca. 10 g) from the transport crates was collected in plastic sample cups with covers.

Additional caecal droppings and caecal content samples from 50 flocks and 50 batches were collected for comparison of analytical methods to detect high levels of *Campylobacter* colonization. This resulted in a total amount of 60 caecal droppings and 60 caecal content samples analyzed.

All samples were collected aseptically, transported to the laboratory under cooled conditions, and analyzed the same day.



**Figure 3.1. Sampling of caecal droppings at broiler farm**



## 2.2 Samples preparation

At the laboratory, the content of each sampling cup (caecal droppings, faecal material and faecal material from crates) was mixed with a wooden spatula. Intestinal packages were removed from plastic bags to collect one caecum, which was immersed in ethanol to remove the external contamination. After ethanol evaporation approximately 1 g of caecal content was collected and pooled per batch.

## 2.3 Enumeration of *Campylobacter* by direct culture method

One gram of all pooled caecal and faecal samples were homogenized with 0.1 % peptone water (Bio-Rad Laboratories, USA) at a ratio of 1:10, plated on Campyfood Agar (CFA; bioMérieux, France) and incubated under microaerobic conditions at 41.5 °C for 48 h. The homogenates of caecal droppings and caecal content samples were further used for enumeration of *Campylobacter* by qPCR and by PMA-qPCR (point 2.4)

Material collected on boot swabs was weighed by comparing the weight of clean and used swabs. Next, 0.1 % peptone water was added to the plastic bag with the boot swabs at a ratio of 1:5. The homogenate was plated and incubated as described above.

Presumptive *Campylobacter* colonies were enumerated and confirmed (at least four per sample) by microscopic observation and by *Campylobacter* species PCR assays (Linton et al., 1996; Vandamme et al., 1997).

## 2.4 Enumeration of *Campylobacter* by qPCR and by PMA-qPCR

From each homogenate of caecal droppings and caecal content samples two aliquots of 0.5 ml were prepared. One aliquot was treated with PMA as described by (Duarte et al., 2015) and the second aliquot was left PMA-untreated. Afterwards, both PMA treated and untreated aliquots were submitted to DNA extraction as described previously (Duarte et al., 2015) and DNA was stored at -20 °C. Next, *Campylobacter* quantification by duplex TaqMan qPCR was performed in both PMA treated and untreated aliquots, but only for samples with countable results by the direct culture method ((Duarte et al., 2015).

For the *Campylobacter* qPCR enumerations a translation of the obtained Ct value to the number of log<sub>10</sub> CFU/g on the basis of the standard curve and dilution factors was made as described by Duarte et al. (2015).

## 2.5 Lateral flow test

Singlepath® Direct Campy Poultry test (Singlepath® test; Merck Millipore, Germany) is a gold labeled immunosorbent assay for the detection of *Campylobacter* positive flocks, colonized with more than 7.5 log<sub>10</sub> cfu/g. The Singlepath® test was applied to all collected caecal droppings (n = 60) and caecal content samples (n = 60). To perform the Singlepath® test, approximately 1 g of sample (prepared as described in "Samples preparation") was analyzed as specified by the manufacturer. Briefly, 9 ml of sample diluent, provided by the manufacturer, was added to the sample, mixed and after sedimentation (10 min) at room temperature, 1 ml of supernatant was heat-inactivated (95 °C) for 15 min, and allowed to cool to room temperature. Next, two drops of sample buffer, provided by the manufacturer, were added to the sample, which was subsequently mixed and allowed to settle for 10 min. Afterwards, 5 drops of the supernatant was applied to the test. Results were visible for qualitative interpretation after 20 min at room temperature.

## 2.6 Data analysis

All *Campylobacter* counts were converted to log<sub>10</sub> values. To test for differences in *Campylobacter* counts between sample types collected at farm level, a Friedman test was used followed by the Wilcoxon signed-rank test with a Bonferroni adjustment. At slaughterhouse level, differences in *Campylobacter* counts between sample types were calculated using the Wilcoxon signed-rank test. Results below the quantification limit (100 cfu/g) were excluded from the analysis.

A linear mixed model, with the method type as a fixed and repeated effect and the batch/flock as the random effect, was used to determine differences in *Campylobacter* counts between quantification methods. Additionally, to define a relation between *Campylobacter* counts obtained by different methods, linear regression analysis was performed.

Since the Singlepath® test is based on immune-chromatographic principles, it produces positive results in the presence of targeted antigen. Therefore, when *Campylobacter* counts measured by direct culture method decreased during samples storage (up to 72 h at 4°C), the Singlepath® test still presented positive results (John et al., 2013). This finding allows using the combination of results from the direct culture and qPCR as proxy gold standard for evaluation of Singlepath® test. Results obtained by the proxy gold standard and by Singlepath® test were compared using McNemar's tests. Additionally, kappa statistics were calculated to measure the agreement between methods.

Described statistical analyses were performed using SPSS software version 22 (IBM Corporation, USA), and a significance level of 5% was used.

### 3. Results

#### 3.1 Comparison of *Campylobacter* counts between sampling types at farm and slaughterhouse level.

At the farm level, in two flocks *Campylobacter* was not detected in any sample type (Table 3.1). The type of sample collected at the farm level had a significant effect on the *Campylobacter* counts ( $P < 0.001$ ), measured by direct plating, with significantly the highest numbers for caecal droppings ( $P < 0.05$ ) and significantly the lowest for boot swabs ( $P < 0.05$ ) in comparison to other sample types.

At the slaughterhouse level, in both caecal content and faecal material samples from six batches *Campylobacter* was not detected (Table 3.1). *Campylobacter* counts in the four remaining batches were significantly higher in caecal content than in faecal material collected from crates ( $P < 0.05$ ).

**Table 3.1. *Campylobacter* counts in caecal droppings, faecal material and boot swabs collected at farm level and in caecal content of slaughtered birds and in faecal material from transport crates collected at slaughterhouse level. For each flock and each batch one sample from every sample type was analyzed.**

Farm				Slaughterhouse		
flock id	<i>Campylobacter</i> counts ( $\log_{10}$ cfu/g)			batch id	<i>Campylobacter</i> counts ( $\log_{10}$ cfu/g)	
	caecal droppings	faecal material	boot swabs		caecal content	faecal material from crates
1	6.53	6.39	5.61	1	9.32	4.69
2	8.32	7.29	6.54	2	9.13	6.97
3	7.88	7.17	5.84	3	< 2	< 2
4	7.12	7.00	6.48	4	< 2	< 2
5	8.28	6.23	5.24	5	8.93	7.40
6	7.29	6.68	4.66	6	< 2	< 2
7	7.39	5.56	5.49	7	< 2	< 2
8	< 2	< 2	< 2	8	< 2	< 2
9	< 2	< 2	< 2	9	7.12	5.96
10	4.81	4.06	3.83	10	< 2	< 2
Mean <sup>1</sup>	7.20 <sup>a</sup>	6.30 <sup>b</sup>	5.46 <sup>c</sup>	Mean <sup>1</sup>	8.63 <sup>A</sup>	6.26 <sup>B</sup>
SD	1.14	1.07	0.90	SD	1.02	1.21

SD – standard deviation

<sup>1</sup>Mean was calculated based on samples above the quantification limit. Different letters indicate significant differences ( $P < 0.05$ ) between samples types. As flocks and batches were not related, *Campylobacter* counts in samples collected at the farm level were not compared with those collected at the slaughterhouse level.

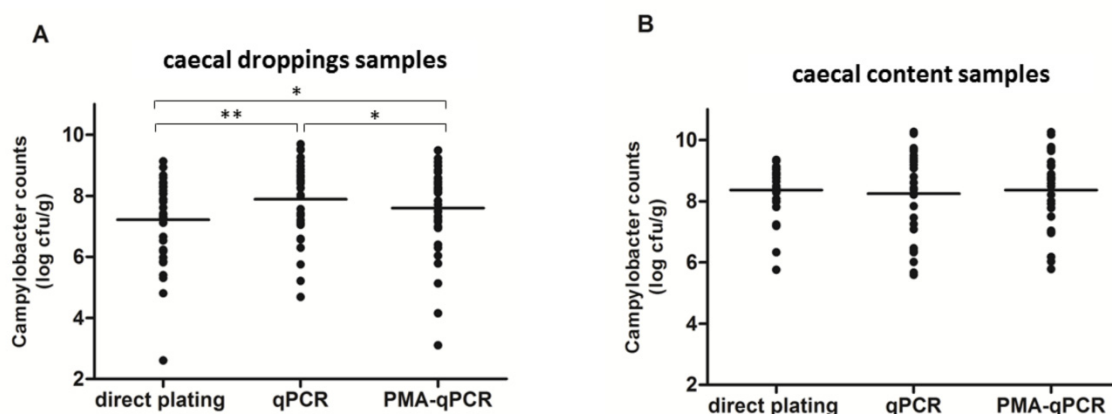
As caecal droppings and caecal content samples showed the highest *Campylobacter* counts among the tested sample types collected at the farm and slaughterhouse level respectively, those types were selected for the evaluation of the analytical methods including the Singlepath® test.

### 3.2 Comparison of *Campylobacter* counts obtained by direct culture, qPCR and PMA-qPCR

The result of the study revealed that *Campylobacter* was recovered by direct culture method in 65 % (39/60) of flocks (caecal droppings) and in 50 % (30/60) of batches (caecal content).

The most commonly recovered species was *Campylobacter jejuni* (63.3 %), followed by *Campylobacter coli* (32.4 %). Further, two flocks were colonized by both *C. jejuni* and *C. coli* and one with *C. lari*.

When comparing *Campylobacter* counts in caecal droppings samples, significantly higher mean counts were recovered by qPCR ( $7.88 \pm 1.18 \log_{10}$  cfu/g) in comparison to PMA-qPCR ( $7.60 \pm 1.40 \log_{10}$  cfu/g;  $P < 0.05$ ) and direct culture ( $7.22 \pm 1.36 \log_{10}$  cfu/g;  $P < 0.01$ ; Fig. 3.2A). Also mean *Campylobacter* counts obtained by PMA-qPCR were significantly higher than those recovered by direct culture ( $P < 0.05$ ; Fig. 2A).



**Figure 3.2.** *Campylobacter* counts in caecal content (A) and in caecal droppings (B) in relation to the quantitative method. Dots and stripes represent individual *Campylobacter* counts and mean values, respectively. Asterisks mark significant differences at  $P < 0.05$  (\*) and  $P < 0.01$  (\*\*).

However, when caecal content samples were analyzed, mean *Campylobacter* counts obtained by direct culture, qPCR and PMA-qPCR accounted for  $8.36 \pm 0.91$ ,  $8.24 \pm 1.41$ ,  $8.36 \pm 1.21 \log_{10}$  cfu/g, respectively, and no significant differences were revealed between methods ( $P > 0.05$ , Fig. 3.2B).

When *Campylobacter* counts obtained by qPCR and PMA-qPCR were plotted against the results of the direct culture method (Fig 3.3), a higher correlation was revealed for sample types treated with PMA (Fig. 3.3C and Fig. 3.3D) in comparison to non-PMA treated samples (Fig. 3.3A and Fig. 3.3B).

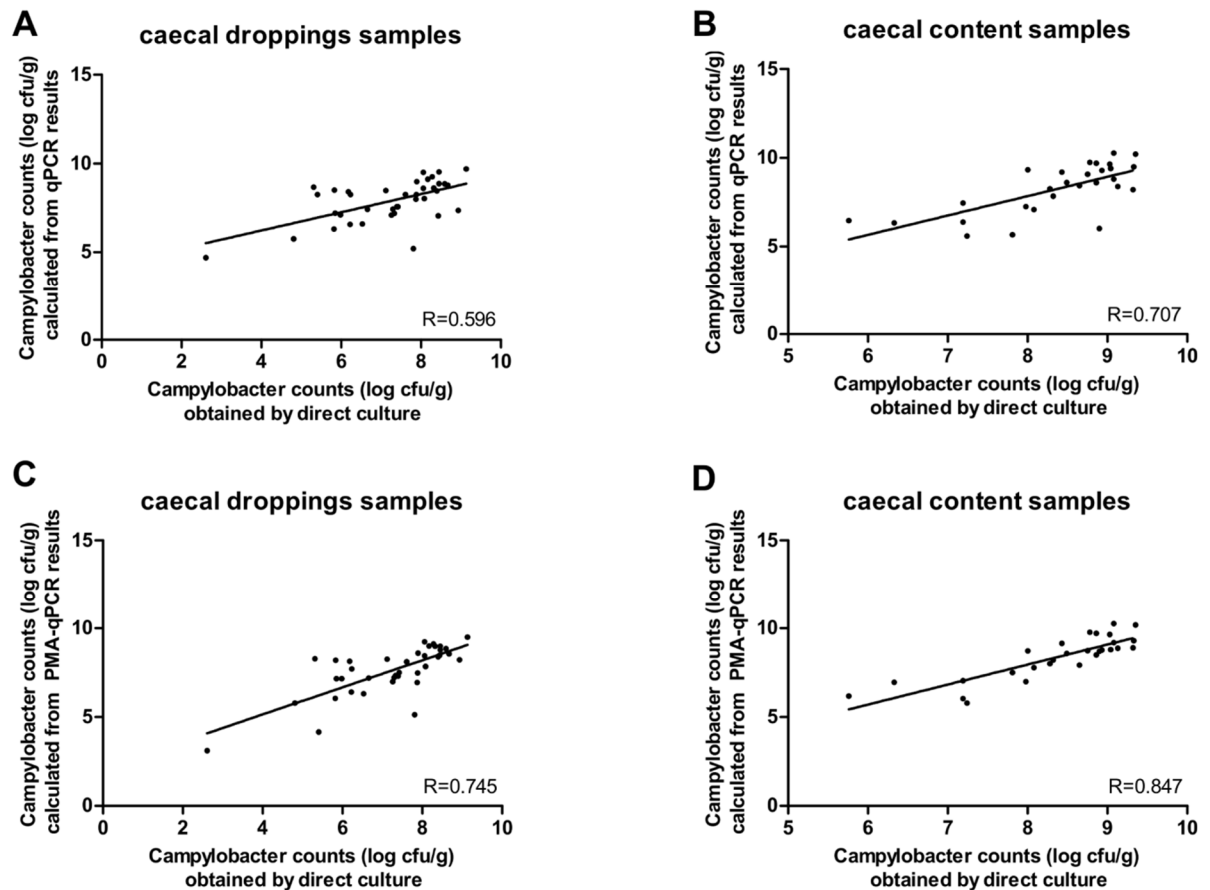


Figure 3.3. Scatter plots demonstrating the correlation between *Campylobacter* counts obtained by direct culture and qPCR in caecal droppings samples (A) and in caecal content samples (B); direct culture and PMA-qPCR in caecal droppings samples (C) and in caecal content samples (D).

### 3.3 Performance of the Singlepath® Direct Campy Poultry test for detection of highly colonized broiler batches

Out of 60 tested flocks (caecal dropping samples), 27 and 26 were identified as colonized with more than  $7.5 \log_{10}$  cfu/g by the Singlepath® test and the proxy gold standard (i.e. combined results of direct culture method and qPCR), respectively. At the slaughterhouse level (caecal content samples), 60 batches were examined. Twenty five were tested positive by the Singlepath® test and 24 were colonized with at least  $7.5 \log_{10}$  cfu/g based on direct culture or qPCR method.

The McNemar's test could not detect significant differences in performance of the Singlepath® test and proxy gold standard for detection *Campylobacter* colonization levels of at least  $7.5 \log_{10}$  cfu/g ( $P>0.05$ ). Additionally, the Kappa value for caecal droppings and caecal content samples was 0.899 ( $P<0.001$ ) and 0.966 ( $P<0.001$ ), respectively, and shows as such high agreement between the Singlepath® test and the proxy gold standard method.

The sensitivity and specificity of the Singlepath® test was calculated using a 2 x 2 table approach (Table 3.2). Of all examined samples, two caecal droppings and one caecal content sample tested positive by Singlepath® test even though *Campylobacter* counts in these samples were lower than 7.5 log<sub>10</sub> cfu/g. Only one false negative outcome of the Singlepath® test was recorded for all tested samples. Based on these results, the sensitivity and specificity of Singlepath® test accounted for 96.15% (95% CI 80.30 – 99.36) and 94.12% (95% CI 80.29 – 99.11), respectively, when caecal droppings samples were analyzed. Higher values were obtained for caecal content samples, namely 100.00% sensitivity (95% CI 85.62 – 100.00) and 97.22% specificity (95% CI 85.42 – 99.54).

**Table 3.2. Performance of the Singlepath® Direct Campy Poultry test in identification of flocks (caecal droppings) at farm level and batches (caecal content) at slaughterhouse level colonized with more than 7.5 log<sub>10</sub> cfu/g.**

caecal droppings			
test <sup>1</sup>	proxy gold standard <sup>2</sup>		
	≤7.5 log <sub>10</sub>	≥7.5 log <sub>10</sub>	total
	cfu/g	cfu/g <sup>3</sup>	
negative	32	1	33
positive	2	25	27
total	34	26	60

caecal content			
test <sup>1</sup>	proxy gold standard		
	≤7.5 log <sub>10</sub>	≥7.5 log <sub>10</sub>	total
	cfu/g	cfu/g	
negative	35	0	35
positive	1	24	25
total	36	24	60

<sup>1</sup>Singlepath® Direct Campy Poultry test.

<sup>2</sup>Combined results of direct culture and qPCR were treated as the proxy gold standard.

<sup>3</sup>*Campylobacter* counts obtained by at least method (direct culture or qPCR) were equal or higher than 7.5 log<sub>10</sub> cfu/g.

## 4. Discussion

First, in the present study different sample types collected at farm and slaughterhouse level were analyzed for *Campylobacter* counts. At farm level, the fact that the highest numbers were recovered in caecal droppings may not be surprising, as caeca are the highest colonized parts of the intestinal tract of birds (Cason et al., 2007). Additionally, caecal droppings may allow better *Campylobacter* survival than faecal material due to possible antimicrobial activities of urate crystals in faeces (Rudi et al., 2004). However, the practical disadvantage of caecal droppings is that they are more difficult to collect in broiler houses than faecal material, as they are less frequently present and harder to be properly recognized (EFSA, 2012b).

The most applicable sample type, already in use for detection of *Salmonella* in broiler flocks, would be boot swab samples. While the sensitivity of boot swabs for *Campylobacter* detection is reported as not being significantly different from the sensitivity of caecal droppings and faecal material (Vidal et al.,

2013), in the present study, this sample type delivered lower *Campylobacter* counts. This might be explained by the fact that when individuals are walking through the broiler house, not only fresh caecal droppings are collected on boot swabs but also bedding materials in which *Campylobacter* is exposed to long lasting dry and oxidative stresses. Similarly, at the slaughterhouse level, higher *Campylobacter* counts found in caecal content samples than in faecal material collected from transporting crates can be explained by factors discussed above.

Second, an evaluation of a direct culture method, qPCR and PMA-qPCR for enumeration of *Campylobacter* on caecal droppings and caecal content samples was performed. No significant differences in *Campylobacter* counts between the analytical methods were observed when working with fresh caecal content samples. In contrast, when caecal droppings samples were analyzed, significantly higher *Campylobacter* counts were recovered by qPCR in comparison to PMA-qPCR or direct plating. This observation suggests that caecal droppings samples contained a fraction of dead campylobacters that is quantified together with viable cells by qPCR but not by direct plating and by PMA-qPCR (Josefsen et al., 2010). Presence of dead *Campylobacter* cells in caecal droppings can be caused by length of the transportation and/or storage time of samples (Rodgers et al., 2012). In contrast, caecal content was retained inside the caecum until the laboratory analyses, which excludes dry and oxidative stress impairing *Campylobacter* survival.

The application of PMA treatment improved the correlation between the direct culture and the qPCR based method (Fig. 4), which was in agreement with a previous report using PMA-qPCR for quantification of *Campylobacter* in artificially and naturally contaminated poultry meat (Seinige et al., 2014). However, a significant difference in *Campylobacter* counts on caecal droppings samples was still observed when comparing the direct culture method and the PMA-qPCR. Optimization experiments confirmed the effectiveness of PMA treatment at a concentration of 25 µg/ml for reduction of signals from membrane-damaged *Campylobacter* cells in carcass rinses (Duarte et al., 2015). However, it was also reported that the use of PMA at a concentration of 25.55 µg/ml is ineffective for complete inhibition of signals from membrane-damaged *Campylobacter* cells in carcass rinses (Pacholewicz et al., 2013; Seinige et al., 2014) and that a concentration of 51.1 µg/ml is more optimal (Seinige et al., 2014). Apart from the PMA concentration, other factors might also influence the qPCR signal reduction when analyzing caecal samples. Therefore, further optimization of the PMA treatment is needed for reliable quantification of *Campylobacter* membrane-intact cells in samples from caecal origin.

Investigation of the birds' *Campylobacter* colonization level is demanding in terms of equipment, personnel and time. Additionally, testing all broiler batches might be not possible due to the logistic reasons. Therefore, the choice of the sample type and the analytical method might be also influenced by the cost of travelling and/or transportation. A possible solution might be the use of the postal service

and qPCR method for quantification of stressed and non-culturable *Campylobacter* in samples which are extensively stored and transported before further analyses.

Since a *Campylobacter* colonization can quickly spread throughout a broiler flock (van Gerwe et al., 2009), testing for *Campylobacter* should be performed as closely as possible to the slaughter day to provide precise information. Therefore, a rapid and reliable test that can be executed by non-laboratory skilled personnel for discrimination between flocks colonized below and above 7.5 log<sub>10</sub> cfu/g is needed. In the present study, the Singlepath® test was evaluated on caecal droppings and caecal content samples. In three samples (2 caecal droppings and 1 caecal content) *Campylobacter* counts for both culture method and qPCR were below the detection limit of the test proposed by the manufacturer (7.5 log<sub>10</sub> cfu/g) although a positive outcome of the Singlepath® test was recorded. It implies that in some cases the Singlepath® test is able to detect also lower *Campylobacter* concentrations than 7.5 log<sub>10</sub> cfu/g. The only false negative result was obtained when the flock was colonized by *C. lari*, which might be detected by the Singlepath® test probably at higher concentrations because the test was designed primarily to detect *C. jejuni* and *C. coli* as the most prevalent *Campylobacter* species in poultry (EFSA, 2010a).

## 5. Conclusions

In conclusion, the sample type and the analytical method used influences quantitative results on *Campylobacter* colonization level.

Caecal droppings or caecal content are the most appropriate sample types for quantification of broilers' *Campylobacter* colonization level.

The direct culture method and qPCR based methods are equally sensitive for *Campylobacter* enumeration in caecal content samples. Application of the PMA treatment before qPCR analyses reduces the detection of dead cells by qPCR. However, qPCR is an attractive alternative for the culture based method for quantification of stressed and non-culturable *Campylobacter* in samples that are extensively stored and transported before further analyses.

The Singlepath® test could be considered an alternative, qualitative method, which can be performed under non-laboratory conditions and allows rapid detection of flocks/batches colonized with at least 7.5 log<sub>10</sub> cfu of *Campylobacter* per gram of caecal content or caecal droppings sample.

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***CAMPYLOBACTER* CARCASS CONTAMINATION  
THROUGHOUT THE SLAUGHTER PROCESS OF  
*CAMPYLOBACTER*-POSITIVE BROILER BATCHES**

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*Seliwiorstow, T., J. Baré, I. Van Damme, M. Uyttendaele, L. De Zutter (2015): Campylobacter Carcass Contamination throughout the Slaughter Process of Campylobacter-positive Broiler Batches. Int. J. Food Microbiol. 194, 25-31.*

## Abstract

*Campylobacter* contamination on broiler carcasses of *Campylobacter* colonized flocks was quantified at seven sampling sites throughout the slaughter process. For this purpose, in four slaughterhouses samples were collected from twelve *Campylobacter* positive batches.

Broilers from all visits carried high numbers of campylobacters in their caeca ( $\geq 7.9 \log_{10}$  cfu/g). *Campylobacter* counts on feathers (up to  $6.8 \log_{10}$  cfu/g), positively associated with the breast skin contamination of incoming birds and carcasses after plucking, were identified as an additional source of carcass contamination. A high variability in *Campylobacter* carcass contamination on breast skin samples within batches and between batches in the same slaughterhouse and between slaughterhouses was observed. In slaughterhouses A, B, C and D *Campylobacter* counts exceeded a limit of 1000 cfu/g on 50%, 56%, 78% and 11% of carcasses after chilling, respectively. This finding indicates that certain slaughterhouses are able to better control *Campylobacter* contamination than others.

Overall, the present study focuses on the descriptive analysis of *Campylobacter* counts in different slaughterhouses, different batches within a slaughterhouse and within a batch at several sampling locations.

## 1. Introduction

*Campylobacter* is the most common cause of bacterial foodborne infection in humans in developed countries (WHO, 2012). According to the European Food Safety Authority report (EFSA, 2014), the notification rate of human campylobacteriosis in 2011 exceeded 50 per 100,000 of population in the European Union. Common clinical symptoms of campylobacteriosis are diarrhea, vomiting, abdominal pain and fever but infection can also lead to severe complications such as Guillain-Barré syndrome (Rees et al., 1995), reactive arthritis (Hannu et al., 2002) and also irritable bowel syndrome (Gradel et al., 2009). Campylobacteriosis in humans is mainly caused by two species: *Campylobacter jejuni* and *Campylobacter coli* (Park, 2002). Poultry are considered the main *Campylobacter* reservoir (Humphrey et al., 2007) and it is estimated that 50-80% of human campylobacteriosis cases may be attributed to the chicken reservoir as a whole, while 20-30% is assumed to be linked to handling, preparation and consumption of broiler meat (EFSA, 2011a). Several risk assessment studies concluded that the reduction of *Campylobacter* numbers on carcasses would lead to lower number of human cases associated with handling and consumption of broiler meat (Rosenquist et al., 2003; Nauta et al., 2005; Uyttendaele et al., 2006; Havelaar et al., 2007).

An effective way to protect public health from *Campylobacter* foodborne infections could be the decrease of the prevalence and numbers of *Campylobacter* in broiler chickens at the primary production stage (EFSA 2010). However, at present, application of stringent general biosecurity interventions at farm level cannot prevent *Campylobacter* infection of broiler flocks at the end of the rearing period (Newell et al., 2011).

It is documented that contamination of carcasses surface with *Campylobacter* occurs when *Campylobacter* positive flocks are slaughtered (Newell et al., 2001; Miwa et al., 2003) and it has been further proven that slaughter of birds with low mean *Campylobacter* colonization levels in their caeca results in lower carcass contamination and consequently in lower public health risk (Reich et al., 2008; Rosenquist et al., 2006). Since carcasses contaminated with high numbers of *Campylobacter* are related with high consumers' health risk (Callicott et al., 2008; Nauta and Havelaar, 2008), quantitative data are in the higher interest of public health than presence-absence testing.

According to EFSA, certain slaughterhouses can better control the *Campylobacter* counts on carcasses than others. This suggests that there is an opportunity for reducing *Campylobacter* numbers during slaughter process (EFSA, 2011). Similarly, a recent study in Belgium showed significant differences in *Campylobacter* counts on carcasses after chilling between 9 slaughterhouses (Habib et al., 2012). Possibly, this variability might be explained by external birds' contamination, intestinal colonization level and by carcass contamination at the earlier slaughter stages.

Thus, the aim of the present study was to provide insights in *Campylobacter* counts on broiler carcasses throughout the slaughter process of *Campylobacter* positive flocks. The study was set-up to take into account the intravariability and intervariability in broiler slaughterhouses.

## **2. Materials and methods**

### **2.1. Slaughterhouse profiles**

Four Belgian slaughterhouses were selected based on data obtained during 2008 EFSA Baseline study. At that moment two of them, slaughterhouses A and C, were identified as slaughterhouses with both high prevalence (65 % and 56 %, respectively) and high percentage of carcasses contaminated with more than 3 log<sub>10</sub> cfu/g (35 % and 32 %, respectively). On the other hand slaughterhouses G and H had lower prevalence of *Campylobacter* (42 % and 36 %, respectively) and also lower percentage of carcasses contaminated with more than 3 log<sub>10</sub> cfu/g (14% and 19%, respectively; Habib et. al., 2012). Selected characteristics for each selected slaughterhouse are summarized in Table 4.1.



**Table 4.1. Selected slaughterhouses' characteristics.**

	Slaughterhouse			
	A	B	C	D
Line speed <sup>a</sup>	11,000	12,700	9,000	12,000
Stunning	Electrical	Electrical	Electrical	Gas
Minimum scalding water temperature <sup>b</sup>	52.65 °C	52.30 °C	52.50 °C	54.70 °C
Maximum scalding water temperature <sup>b</sup>	53.70 °C	53.08 °C	53.70 °C	54.95 °C
Scalding time	150 s	250 s	138 s	145 s
Plucking time	35 s	42 s	43 s	42 s
Final inside-outside washer	Present	Present	Not present	Present
Water chilling tanks	Not present	Not present	Present	Not present
Water chilling temperature <sup>c</sup>	/	/	Tank 1 - 8.85 °C Tank 2 - 6.57 °C Tank 3 - 3.90 °C	/
Water sprays in chiller	Not present	Present	Present	Not present
Chilling time	105 min	120 min	135 min <sup>d</sup>	150 min
Air temperature in chiller	From 2.1 to 3 °C	From 3 to 5 °C	From 0 to 2 °C	From -2 to 0 °C
Carcasses temperature after chilling	From 2 to 4 °C	From 6 to 11 °C	From 5 to 6 °C	From 2 to 3 °C
<i>Campylobacter</i> positive samples <sup>e</sup>	56%	65%	42%	36%
Highly contaminated samples ( $\geq 3 \log_{10}$ cfu/g) <sup>e</sup>	32%	35%	14%	19%

<sup>a</sup>Carcasses per hour.

<sup>b</sup>Average from the beginning and the end of each scalding tank measured once during each visit.

<sup>c</sup>Average from the beginning and the end of the chilling tank measured once during each visit.

<sup>d</sup>Including water chilling.

<sup>e</sup>Habib et al. (2012).

## 2.2 Identification of *Campylobacter* positive broiler flocks

Industrially reared flocks, namely a group of more than 12 thousands birds at 6 weeks of age reared in the same broiler house, were selected from the slaughter plan provided by the slaughterhouses. To identify *Campylobacter* positive broiler flocks, caecal droppings were collected at the farm three days before the scheduled slaughter day and analysed for the presence of *Campylobacter* by spreading collected caecal droppings on modified Cefaperazone Charcoal Desoxycholate Agar (mCCDA; *Campylobacter* blood free selective medium CMO739 plus selective supplement SR0155H [Oxoid, England]). Plates were incubated under microaerobic conditions at 41.5°C for 24 hours. Presumptive *Campylobacter* colonies were confirmed by Gram staining and microscopic observation. Birds from a *Campylobacter* positive broiler flock were further sampled during the slaughter process.

## 2.3 Sampling strategy at the slaughterhouse

In each of the four slaughterhouses, three batches (i.e. birds from one flock slaughtered at the same day) were sampled, resulting in 12 visits in the period from February to November 2011.

During each visit six broiler carcasses were collected at each of the following sampling sites: after bleeding, after plucking, after evisceration, after crop puller, before and after washing (i.e. before and after final inside-outside washer), and after chilling (Table 4.2).

**Table 4.2. Sampling sites and sample types collected at the broiler slaughter line during every visit.**

Sampling site	Sample type
After-bleeding	Breast skin; n=6 Breast feathers; n=6
After plucking	Breast skin; n=6
After evisceration	Breast skin; n=6 Caecal content; n=6 Duodenal content; n=6
After crop puller	Breast skin; n=6
Before washing <sup>a</sup>	Breast skin; n=6
After washing <sup>b</sup>	Breast skin; n=6
After chilling	Breast skin; n=6

<sup>a</sup> Before chilling tanks for slaughterhouse C

<sup>b</sup> After chilling tanks for slaughterhouse C

Practical limitations hampered the sampling of carcasses after scalding. In slaughterhouse C, no final inside-outside washer was applied (Table 2). In this slaughterhouse, six carcass samples were taken before and after chilling tanks. Additionally, during every visit six intestinal packages were collected after the evisceration process (Table 2). The first sample at each sampling site was taken not earlier than 10 minutes after the beginning of the investigated batch. Further, sample collection was performed in a consecutive way over one hour of slaughter. All samples were placed in sterile plastic bags, cooled on-site, transported to the laboratory under cooled conditions and analysed the same day.

## 2.4 Sample preparation and enumeration of *Campylobacter*

From carcasses after bleeding, ca. 10 g breast feathers were manually removed and analyzed separately. Next, from each carcass, ca. 10 g of breast skin was sampled for *Campylobacter* enumeration (Baré et al., 2013). From each intestinal package, one cecum and one duodenum was collected, immersed in

ethanol and, after evaporation of the ethanol, approximately 1 g of content was pulled out into sterile plastic bags. All samples were homogenized with 0.1% peptone water (Bio-Rad Laboratories, Inc., Hercules, California, USA) at a ratio of 1:10, plated on CampyFood Agar (CFA; bioMérieux, France; Habib et al., 2011; Ugarte-Ruiz et al., 2012) and incubated under microaerobic conditions at 41.5°C for 48h. After incubation, colonies with typical *Campylobacter* morphology were counted and at least four of them per sample were confirmed was performed by microscopic observation and by PCR (Vandamme et al. 1997).

## 2.5. Data analysis

Bacterial counts were  $\log_{10}$ -transformed to approximate the results to a normal distribution, which was further confirmed by a Shapiro–Wilk test. The detection limit of enumeration was 10 cfu/g for skin and feather samples and 100 cfu/g for caecal and duodenal samples. For samples which were below the enumeration limit, data were set to one-half of the enumeration threshold (Rosenquist et al., 2006). Statistical analyses were carried out using commercial software (Stata/MP 12.1 StataCorp LP, College Station, TX). A significance level of 5% was used. Differences in *Campylobacter* counts between sampling sites were examined per batch using a general linear model. The same method was used to define differences between batches per sampling site within each slaughterhouse. Differences in *Campylobacter* contamination between slaughterhouses at every sampling site were analysed using a random-effects generalized least squares regression, including batch as random variable. Bonferroni adjustments were applied for multiple testing. Pearson correlation coefficients were calculated to determine the association of *Campylobacter* counts between selected sampling sites.

## 3. Results

For the quantitative monitoring of *Campylobacter* spp. during slaughter, 790 samples (encompassing carcass, caecal, duodenal and feather samples) from 12 *Campylobacter* positive batches slaughtered in four Belgian slaughterhouses were collected and analyzed for the number of *Campylobacter* present.

### 3.1 Internal and external *Campylobacter* carriage of incoming birds

The colonization level of caeca and duodenum was determined to define the internal *Campylobacter* carriage. In addition, *Campylobacter* feathers contamination of incoming birds (in the present study sampled after bleeding) illustrated the initial external contamination before slaughter.

As only *Campylobacter* colonized chickens were selected for this study, all caecal content samples were positive for *Campylobacter* (Table 4.3).

**Table 4.3. *Campylobacter* counts (a) and detection data (b) from caeca, duodenum and feathers samples, collected in four slaughterhouses.**

Slaughterhouse	Batch	caeca		duodenum		feathers	
		a <sup>1</sup>	b <sup>2</sup>	a <sup>1</sup>	b <sup>2</sup>	a <sup>1</sup>	b <sup>2</sup>
A	1	9.48 ± 0.25	6/6	*3.37 ± 1.32	4/6	3.13 ± 1.26	5/6
	2	*8.52 ± 1.14	6/6	1,70 <sup>3</sup>	0/6	1.42 ± 1.78	1/6
	3	9.59 ± 0.16	6/6	1.85 ± 0.37	1/6	2.77 ± 1.87	5/6
B	1	*9.28 ± 0,43	6/6	*6.26 ± 0.93	6/6	5.70 ± 0.25	6/6
	2	8.13 ± 0,47	6/6	4.71 ± 0.93	6/6	5.03 ± 0.52	6/6
	3	7.99 ± 0,90	6/6	5.00 ± 1.05	6/6	3.46 ± 1.45	5/6
C	1	9.71 ± 0.29	6/6	5.23 ± 0.73	6/6	6.80 ± 0.36	6/6
	2	9,28 ± 0.37	6/6	4.49 ± 0.69	6/6	5.49 ± 0.87	5/6
	3	*8.06 ± 0.88	6/6	3.82 ± 1.39	6/6	*3.79 ± 2.40	4/6
D	1	*9.71 ± 0.20	6/6	*2.97 ± 1.50	3/6	*2.73 ± 1.74	4/6
	2	8.71 ± 0.36	6/6	5.45 ± 0.52	6/6	5.71 ± 0.62	6/6
	3	8.93 ± 0.55	6/6	5.52 ± 0.84	6/6	5.86 ± 0.58	6/6

<sup>1</sup>log<sub>10</sub> cfu/g ± SD

<sup>2</sup>*Campylobacter* positive samples/total tested samples

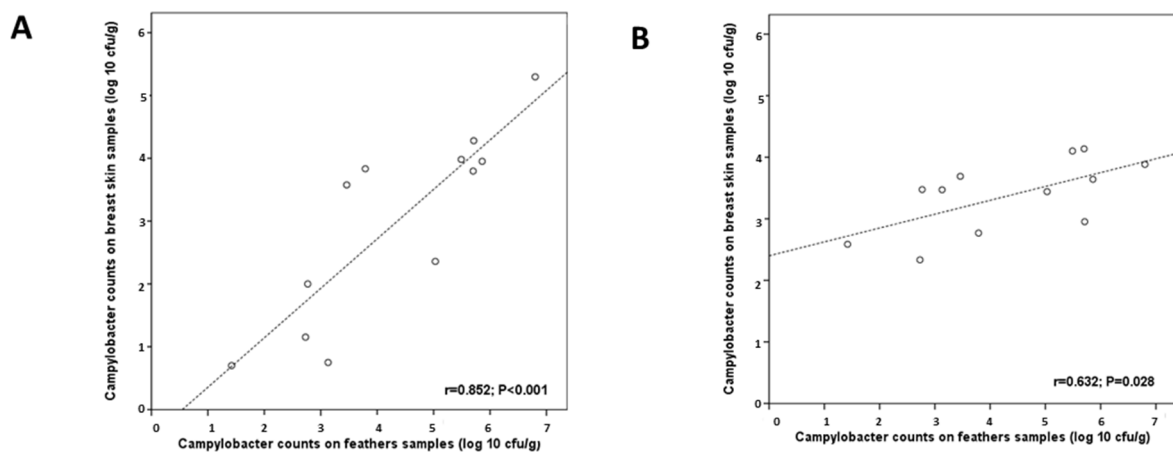
<sup>3</sup>half of the detection limit

\*significant difference ( $P < 0.05$ ) in *Campylobacter* counts between batches within a slaughterhouse

*Campylobacter* colonization level varied significantly between batches ( $P < 0.05$ ) within a slaughterhouse, but not between slaughterhouses ( $P > 0.05$ ). Significant correlations in *Campylobacter* counts between caecal samples and breast skin samples collected at the various sampling sites were not observed ( $P > 0.05$ ). Notwithstanding all caecal content samples were *Campylobacter* positive, *Campylobacter* was recovered from 0 to 100% of the duodenal samples of these batches (Table 4.3). The average *Campylobacter* counts in the positive duodenum samples were always significantly lower ( $P < 0.001$ ) than *Campylobacter* counts in the corresponding caecal content. Using a Pearson's correlation coefficient test, no significant correlation between *Campylobacter* counts in caeca and duodenum was found. The duodenal concentration of *Campylobacter* varies significantly between batches in slaughterhouses A, B

and D ( $P<0.05$ ; Table 4.3). Similarly to the caecal samples, no significant correlation was present between *Campylobacter* counts in duodenum and on breast skin samples ( $P>0.05$ ).

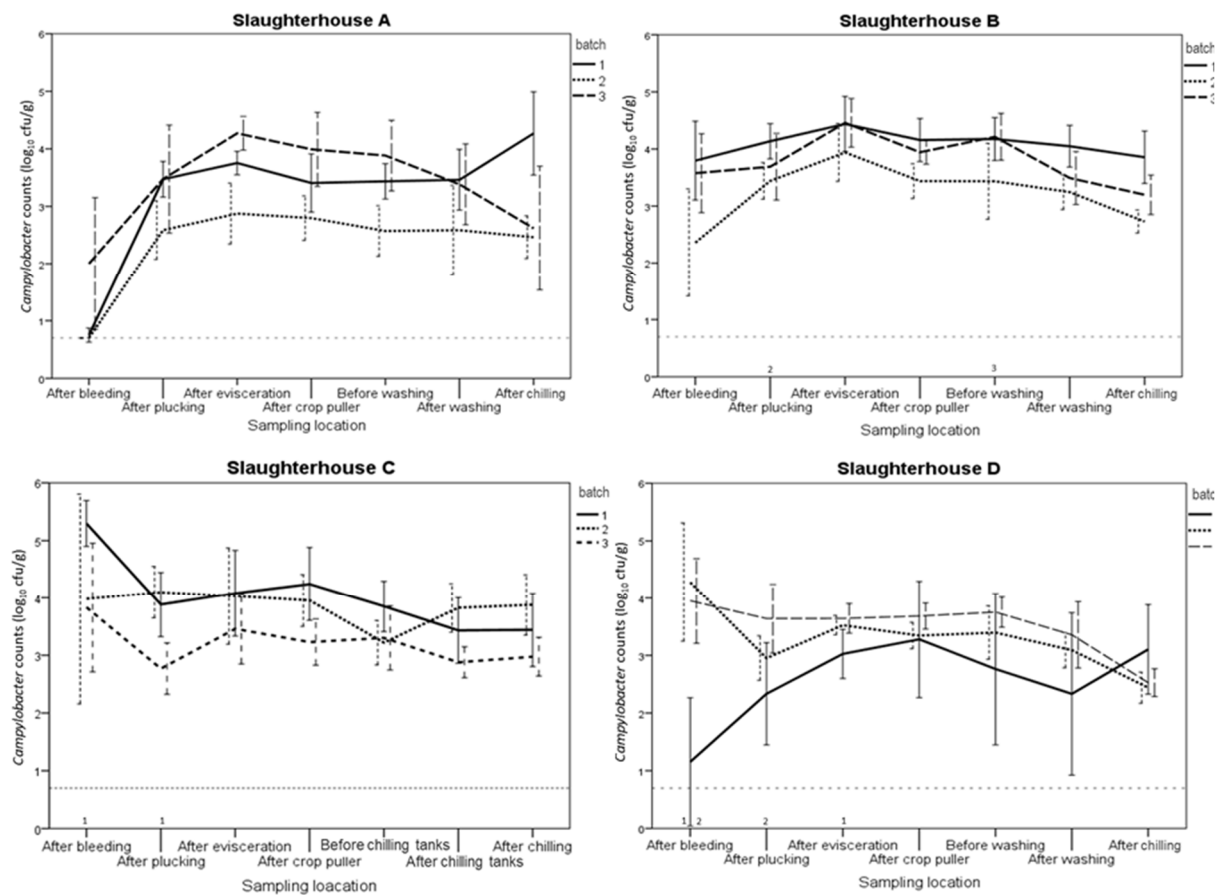
Concerning feather samples, *Campylobacter* was detected at least in one sample per batch. The *Campylobacter* counts on feathers were significantly lower in slaughterhouse A than in the other slaughterhouses ( $P<0.05$ ) and no significant differences were observed between slaughterhouses B, C and D ( $P>0.05$ ). Only in slaughterhouses C and D, *Campylobacter* counts on feathers differed significantly between batches ( $P<0.05$ ; Table 3). *Campylobacter* counts on feathers were significantly associated with counts on breast skin samples collected after bleeding and after plucking (Figure 4.1).



**Figure 4.1.** Relationship between *Campylobacter* counts on breast feathers and breast skin samples collected from carcasses after bleeding (A) and after plucking (B). Each dot represents the mean value of one batch.

### 3.2 *Campylobacter* contamination of carcasses during the slaughter process

Enumeration of *Campylobacter* throughout the slaughter process allowed assessing the role of each slaughter step in the *Campylobacter* carcass contamination. Mean *Campylobacter* counts at each sampling site, plotted per batch (Figure 4.2), revealed that the impact of the slaughter process on the *Campylobacter* carcasses contamination differed between slaughterhouses and also between batches within the same slaughterhouse.



**Figure 4.2.** *Campylobacter* counts (mean log<sub>10</sub> cfu/g  $\pm$  SD) on carcass breast skin throughout the slaughter line in four slaughterhouses (A-D) during the slaughter of *Campylobacter* positive batches. The horizontal dashed line indicates half of the enumeration limit.

### 3.2.1 Intra-slaughterhouse variability in four slaughterhouses

In slaughterhouse A, *Campylobacter* was detected on carcasses after bleeding in six out of eighteen samples (Table 4.4). However, all breast skin samples collected after plucking and at following sampling locations were positive for *Campylobacter*.

Average *Campylobacter* counts on carcasses after bleeding in slaughterhouse A for the first and the second batches were lower than 1 log<sub>10</sub> cfu/g (Table 4.4). Nevertheless, for both these batches a significant increase in *Campylobacter* counts was observed when comparing carcasses after bleeding to carcasses after plucking, after evisceration and after chilling ( $P < 0.001$ ; Table 4.5).

**Table 4.4. *Campylobacter* counts (a) and detection data (b) on carcasses collected after bleeding, after plucking, after evisceration and after chilling in four slaughterhouses.**

Slaughterhouse	Batch	After bleeding		After plucking		After evisceration		After chilling	
		a <sup>1</sup>	b <sup>2</sup>	a <sup>1</sup>	b <sup>2</sup>	a <sup>1</sup>	b <sup>2</sup>	a <sup>1</sup>	b <sup>2</sup>
A	1	<sup>1</sup> 0.75 ± 0.12	1/6	<sup>1</sup> 3.47 ± 0.31	6/6	<sup>1</sup> 3.75 ± 0.20	6/6	<sup>1</sup> 4.27 ± 0.72	6/6
	2	<sup>1</sup> 0.70 <sup>4</sup>	0/6	<sup>1</sup> 2.59 ± 0.51	6/6	<sup>1</sup> 2.88 ± 0.53	6/6	<sup>1</sup> 2.47 ± 0.37	6/6
	3	<sup>1</sup> 2.00 ± 1.15	5/6	<sup>1</sup> 3.48 ± 0.94	6/6	<sup>1</sup> 4.27 ± 0.29	6/6	<sup>1</sup> 2.62 ± 1.08	6/6
	m <sup>3</sup>	1.15 ± 0.88	6/18	3.18 ± 0.74	18/18	3.63 ± 0.69	18/18	3.12 ± 1.11	18/18
B	1	<sup>1</sup> 3.80 ± 0.69	6/6	<sup>1</sup> 4.14 ± 0.31	6/6	<sup>1</sup> 4.44 ± 0.48	6/6	<sup>1</sup> 3.85 ± 0.46	6/6
	2	<sup>1</sup> 2.36 ± 0.94	6/6	<sup>1</sup> 3.44 ± 0.32	6/6	<sup>1</sup> 3.94 ± 0.51	6/6	<sup>1</sup> 2.73 ± 0.20	6/6
	3	<sup>1</sup> 3.58 ± 0.69	6/6	<sup>1, 1</sup> 3.69 ± 0.58	6/6	<sup>1</sup> 4.46 ± 0.42	6/6	<sup>1</sup> 3.20 ± 0.34	6/6
	m <sup>3</sup>	3.24 ± 0.98	18/18	3.76 ± 0.50	18/18	4.28 ± 0.51	18/18	3.26 ± 0.58	18/18
C	1	<sup>1</sup> 5.29 ± 0.40	6/6	<sup>1</sup> 3.88 ± 0.56	6/6	<sup>1</sup> 4.08 ± 0.75	6/6	<sup>1</sup> 3.44 ± 0.64	6/6
	2	<sup>1</sup> 3.98 ± 1.82	5/6	<sup>1</sup> 4.10 ± 0.45	6/6	<sup>1</sup> 4.03 ± 0.84	6/6	<sup>1, 1</sup> 3.88 ± 0.53	6/6
	3	<sup>1</sup> 3.83 ± 1.12	6/6	<sup>1</sup> 2.77 ± 0.44	6/6	<sup>1</sup> 3.46 ± 0.61	6/6	<sup>1</sup> 2.97 ± 0.33	6/6
	m <sup>3</sup>	4.37 ± 1.36	18/18	3.58 ± 0.76	18/18	3.86 ± 0.75	18/18	3.43 ± 0.62	18/18
D	1	<sup>1</sup> 1.15 ± 1.11	1/6	<sup>1</sup> 2.33 ± 0.89	6/6	<sup>1</sup> 3.03 ± 0.43	6/6	<sup>1</sup> 3.10 ± 0.78	6/6
	2	<sup>1</sup> 4.28 ± 1.03	6/6	<sup>1, 1</sup> 2.95 ± 0.38	6/6	<sup>1</sup> 3.52 ± 0.17	6/6	<sup>1</sup> 2.44 ± 0.27	6/6
	3	<sup>1</sup> 3.95 ± 0.74	6/6	<sup>1</sup> 3.64 ± 0.60	6/6	<sup>1</sup> 3.64 ± 0.25	6/6	<sup>1</sup> 2.53 ± 0.24	6/6
	m <sup>3</sup>	3.13 ± 1.71	13/18	2.98 ± 0.83	18/18	3.40 ± 0.39	18/18	2.69 ± 0.56	18/18

<sup>1</sup>log<sub>10</sub> cfu/g ± SD

<sup>2</sup>*Campylobacter* positive samples/total tested samples

<sup>3</sup>mean *Campylobacter* counts per slaughterhouse

<sup>4</sup>half of the detection limit

<sup>1, 1, 1</sup>different roman numerals indicate significant difference ( $P < 0.05$ ) in *Campylobacter* counts between batches within a slaughterhouse at the certain sampling site when comparing mean log *Campylobacter* counts

After bleeding contamination of broilers from the third batch was significantly higher than contamination of birds from the other two batches ( $P < 0.01$ ). Additionally, during slaughter of the third batch, *Campylobacter* counts after evisceration were significantly higher than carcass contamination after bleeding and after chilling ( $P < 0.01$ ; Table 4.5).

Surprisingly, chilling caused a significant increase ( $P < 0.001$ ) of the carcasses contamination during the slaughter of the first batch (Table 5). After chilling *Campylobacter* counts on carcasses from this batch were significantly higher ( $P < 0.001$ ) than counts on carcasses from the second and the third batches collected at the same sampling location (Table 4.4).

*Campylobacter* was recovered from all breast skin samples collected throughout the slaughter process in slaughterhouse B.

During the slaughter of the first batch, no significant changes in the carcasses contamination were detected between all sampling sites ( $P > 0.05$ ). On the contrary, *Campylobacter* counts varied significantly

between certain sampling sites when the second and the third batch were slaughtered as indicated in Table 4.5.

*Campylobacter* carcass contamination in batch 2 significantly increased after plucking ( $P<0.05$ ) compared to after bleeding. This increase was followed by a significant decrease in contamination between carcasses after evisceration and after chilling ( $P<0.01$ ). During slaughter of the third batch, a significant increase in *Campylobacter* counts was observed between carcasses after bleeding and after evisceration. Washing and chilling significantly reduced the carcass contamination in this batch.

Broilers from the second batch carried significantly ( $P<0.05$ ) lower numbers of *Campylobacter* after bleeding and after chilling in comparison to birds from the first and the third batches (Table 4.4).

In slaughterhouse C, the *Campylobacter* was detected in all breast skin samples, except for one carcass collected after bleeding from the second batch.

*Campylobacter* carcass contamination after bleeding in slaughterhouse C varied not significantly between batches. *Campylobacter* counts on carcasses after bleeding were significantly lower ( $P<0.01$ ) than on carcasses after plucking when the first batch was slaughtered. The following slaughter steps did not significantly influence carcass contamination. Likewise, during the slaughter of the second and the third batches no significant changes between sampling sites were observed ( $P>0.05$ ; Table 4.5).

In slaughterhouse D, *Campylobacter* was not recovered from five samples from batch one collected at the beginning of the line (after bleeding; Table 4.4). Consequently, *Campylobacter* counts were significantly lower ( $P<0.001$ ) on carcasses from the first batch compared to the other two batches at this sampling site. As illustrated in Table 4.5, no significant changes ( $P>0.05$ ) were observed in carcasses contamination on the first three sampling sites when the third batch was slaughtered. A significant reduction in *Campylobacter* numbers was obtained in the second batch between carcasses after bleeding and after plucking ( $P<0.01$ ). In contrast, when the first batch was slaughtered, carcasses collected after evisceration carried significantly higher *Campylobacter* counts than carcasses after bleeding ( $P<0.05$ ). A significant decrease in *Campylobacter* counts was observed when comparing the carcass contamination before washing and after chilling in batches two and three ( $P<0.05$ ). On the other hand, no significant effect of washing and chilling was observed when the first batch was slaughtered



**Table 4.5. Differences in *Campylobacter* counts between selected sampling locations. Grey table cells with black numbers indicate significant increase in *Campylobacter* counts between two locations. Black cells with white numbers indicate significant decrease in *Campylobacter* counts. P-values below 0.05 are bolded.**

slaughterhouse		A						B					
batch		1		2		3		1		2		3	
		a	b	a	b	a	b	a	b	a	b	a	b
<b>comparison</b>													
after bleeding – after plucking		+ 2.72	<0.001	+ 1.89	<0.001	+ 1.48	0.053	+ 0.34	0.846	+ 1.08	0.016	+ 0.11	1.000
after bleeding – after evisceration		+ 3.00	<0.001	+ 2.18	<0.001	+ 2.27	0.001	+ 0.64	0.205	+ 1.58	<0.001	+ 0.88	0.037
after bleeding – after chilling		+ 3.52	<0.001	+ 1.76	<0.001	+ 0.62	0.847	+ 0.06	1.000	+ 0.37	0.878	- 0.38	0.801
after plucking – after evisceration		+ 0.28	0.914	+ 0.29	0.942	+ 0.80	0.638	+ 0.30	0.905	+ 0.50	0.653	+ 0.77	0.097
after plucking – after chilling		+ 0.80	0.042	- 0.12	0.999	- 0.86	0.556	- 0.28	0.930	- 0.71	0.249	- 0.49	0.549
after evisceration – after chilling		+ 0.52	0.394	- 0.41	0.756	- 1.65	0.021	- 0.58	0.303	- 1.21	0.005	- 1.26	0.001
before washing - after chilling		+ 0.83	0.030	- 0.10	1.000	- 1.27	0.139	- 0.32	0.879	- 0.71	0.258	- 1.02	0.011
after washing - after chilling		+ 0.81	0.038	- 0.12	0.999	- 0.77	0.678	- 0.19	0.989	- 0.52	0.619	- 0.29	0.929
slaughterhouse		C						D					
batch		1		2		3		1		2		3	
		a	b	a	b	a	b	a	b	a	b	a	b
<b>comparison</b>													
after bleeding – after plucking		- 1.41	0.003	+ 0.12	1.000	- 1.07	0.053	+ 1.18	0.452	- 1.33	0.001	- 0.31	0.903
after bleeding – after evisceration		- 1.21	0.015	+ 0.05	1.000	- 0.38	0.926	+ 1.87	0.049	- 0.76	0.136	- 0.31	0.905
after bleeding – after chilling		- 1.86	<0.001	- 0.10	1.000	- 0.86	0.191	+ 1.95	0.037	- 1.84	<0.001	- 1.43	<0.001
after plucking – after evisceration		+ 0.20	0.997	- 0.07	1.000	+ 0.69	0.427	+ 0.69	0.906	+ 0.57	0.420	0.00	1.000
after plucking – after chilling		- 0.44	0.840	- 0.22	0.999	+ 0.21	0.996	+ 0.77	0.853	- 0.51	0.547	- 1.12	0.003
after evisceration – after chilling		- 0.64	0.486	- 0.15	1.000	+ 0.48	0.794	+ 0.08	1.000	- 1.09	0.008	- 1.12	0.003
before washing - after chilling		- 0.41	0.881	+ 0.65	0.827	- 0.41	0.881	+ 0.38	0.997	- 0.96	0.027	- 1.23	0.001
after washing - after chilling		+ 0.01	1.000	+ 0.05	1.000	+ 0.10	1.000	+ 0.77	0.853	- 0.65	0.270	- 0.83	0.50

a – difference in *Campylobacter* counts between two indicated locations (log<sub>10</sub> cfu/g). "+" and "-" signs indicate increase and decrease in *Campylobacter* counts, respectively; b – P-value associated with the indicated comparison

### 3.2.2 Inter-slaughterhouse variability

When comparing *Campylobacter* carcass contamination between slaughterhouses (Table 4.4), significantly the highest variability in *Campylobacter* carcass contamination was detected at the beginning of the slaughter process. At this point, the *Campylobacter* counts were significantly lower in slaughterhouse A in comparison to the other slaughterhouses ( $P<0.05$ ). The observed variation in *Campylobacter* concentration between slaughterhouses B, C and D at this stage of the process was not significant ( $P>0.05$ ).

Differences between the slaughterhouses in *Campylobacter* counts observed after plucking were not significant ( $P>0.05$ ). In contrast, after the evisceration process, the breast skin contamination was the lowest in company D and the highest in company B, which resulted in significant differences between both slaughterhouses ( $P<0.05$ ).

Differences between slaughterhouses in *Campylobacter* counts on carcasses after chilling were not significant ( $P>0.05$ ). However, the number of highly contaminated carcasses ( $>1\ 000$  cfu/g) differed between companies. On 9, 10, 14 and 2 carcasses after chilling a limit of 1000 cfu of *Campylobacter* per gram was exceeded in slaughterhouses A, B, C and D, respectively.

A comparison of initial (after bleeding) and final (after chilling) *Campylobacter* carcass contamination revealed that in slaughterhouse A, carcass contamination after chilling was significantly higher ( $P<0.001$ ) than after bleeding when the first and the second batch were slaughtered. *Campylobacter* counts on carcasses after bleeding and after chilling in slaughterhouses B and C were not significantly different ( $P>0.05$ ) with the exception of the first batch in slaughterhouse C, where significant reduction was observed ( $P<0.001$ ). In slaughterhouse D *Campylobacter* counts were reduced ( $P<0.001$ ) during slaughter of batches 2 and 3. However, when the first batch was processed, carcasses after chilling carried significantly higher *Campylobacter* numbers than after bleeding ( $P<0.05$ ).

## 4. Discussion:

This study presents *Campylobacter* counts on broiler carcasses collected in the four slaughterhouses during the slaughter of *Campylobacter* positive batches. When assessing the public health risk, measurement of *Campylobacter* counts seem to be a better estimate than a presence-absence testing. A mathematical model (Nauta and Havelaar, 2008) as well as an observational study (Callicott et al., 2008) showed that the risk of human campylobacteriosis is associated with highly contaminated broiler carcasses. It is also calculated that more than 50% reduction of the public health risk could be achieved if all batches would comply with a limit of 1000 cfu/g neck and breast skin (EFSA, 2011a).

In our study the number of carcasses after chilling contaminated with more than 1000 cfu of *Campylobacter* per gram differed between slaughterhouses showing that certain slaughterhouses are able to produce lower numbers of highly contaminated carcasses than others. This conclusion is consistent with results reported by Habib et al. (2012), who analyzed Belgian survey data from the European Union baseline study. In the present study slaughterhouses A, B and C had a higher percentage of highly contaminated carcasses ( $>3 \log \log_{10} \text{ cfu/g}$ ) than in the baseline study (Habib et al., 2012). This can be explained by the fact that in the present study samples were collected only during the slaughter of *Campylobacter* colonized batches. The only slaughterhouses with the lower percentage of highly contaminated carcasses ( $>3 \log \log_{10} \text{ cfu/g}$ ) in the present study was slaughterhouse D that may suggest that slaughterhouse D is able to better control *Campylobacter* contamination during the slaughter than the other slaughterhouses.

The EU-wide report stated also that the risk for high *Campylobacter* counts on carcasses after chilling varied significantly between slaughterhouses (EFSA, 2010a). To explain the variability in *Campylobacter* contamination levels on carcasses after chilling between slaughterhouses, monitoring of *Campylobacter* counts at seven sampling sites during the slaughter process was performed. Obtained results revealed that the plucking, evisceration, washing and chilling activities all seemed to have an impact on the levels of *Campylobacter* contamination on carcasses.

The trend in *Campylobacter* counts observed after plucking and evisceration depends on the numbers recovered at the beginning of the slaughter process (carcasses after bleeding). During the slaughter of batches with high initial external contamination of *Campylobacter*, the effect of the plucking and evisceration processes on *Campylobacter* carcass contamination was less pronounced. However, the impact of both processes was noticeable when batches with low *Campylobacter* numbers at the beginning of the process were slaughtered. This observation might explain contradictory reports of authors who observed decrease (Allen et al., 2007; Hinton et al., 2004; Klein et al., 2007; Reich et al., 2008), increase (Rosenquist et al., 2006) or no difference (Berrang and Dickens, 2000) in *Campylobacter* counts after evisceration.

Results obtained during the present study further show that washing and chilling separately did not result in a significant reduction in *Campylobacter* counts. Only when comparing carcass contamination before washing and after chilling a significant decrease in *Campylobacter* counts was observed in slaughterhouses D (two batches) and B (one batch). In these two slaughterhouses the chilling time was longer than in slaughterhouse A and the water immersion chilling was not applied. These findings are supported by the study of Allen et al. (2007) who demonstrated that chilling time might be an important factor in the reduction of *Campylobacter* counts.

In slaughterhouse C where water immersion chilling was implemented, *Campylobacter* contamination was not reduced in any of the batches. Also the study by Huezo et al. (2007) indicated that immersion

chilling has a lower impact on *Campylobacter* counts than air chilling if chemical treatment is not applied. On the other hand, in a Danish study both air and water chilling had similar effect on *Campylobacter* counts (Rosenquist et al., 2006).

As suggested by Stern and Robach (2003) and by Hansson et al. (2010), high intra- and inter- batch variability in the *Campylobacter* carcass contamination might be also caused by the variability in the level of caecal colonization. However, in the present study a significant correlation between *Campylobacter* counts in caecal content and on carcasses was not detected. This is in accordance with the finding of Allen et al. (2007) who concluded that the lack of association between numbers of *Campylobacter* in feces and on sampled carcasses might be explained by the variable number of visceral breakage or leakage during evisceration or by inconsistent sampling. Reich et al. (2008) and Rosenquist et al. (2006) on the other hand related *Campylobacter* counts in feces to carcass contamination although they sampled not only highly colonized flocks ( $> 7.5 \log_{10}$  cfu/g) that possibly could influence the results of analysis.

Additionally, the present study shows an association between feathers and skin contamination on birds entering the slaughter process (after bleeding). Scalding at temperature between 52 and 55 °C allowing survival of *Campylobacter* during scalding and consequently contamination of feathers was related also with carcass contamination after plucking. This observation allows the assumption to be made that increase in external contamination of birds entering the slaughter process results in higher carcass contamination.

In conclusion, in the present study high variability in *Campylobacter* counts within batches and between batches, as well as between slaughterhouses was demonstrated. This suggests that a well-designed sampling plan is crucial for accurate estimation of contamination level in broiler batches.

For batches with high variability in counts among individual samples, sample pooling might result in underestimation of *Campylobacter* counts (Vidal et al., 2013) although when analyzing limited number of individual samples ( $n = 3$ ) the probability of acceptance of the batch can increase proportionally to the standard deviation in *Campylobacter* counts (Comin et al., 2014). Additionally, it was demonstrated that control of external contamination of broilers entering the slaughter process may result in lower carcass contamination. Further, our results showed that changes in *Campylobacter* carcass contamination during plucking, evisceration, washing and chilling are batch and slaughterhouse dependent. The identification of factors which drive these differences might be crucial for the controlling of *Campylobacter* at the slaughterhouse level.

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**TRANSFER AND STABILITY OF THE *CAMPYLOBACTER*  
COUNTS ON BROILER CARCASSES DURING SUCCESSIVE  
SLAUGHTER OF BROILER BATCHES WITH A DIFFERENT  
*CAMPYLOBACTER* STATUS**

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## Abstract

In the present study the dynamics of *Campylobacter* numbers on carcasses after scalding/bleeding, plucking, evisceration and washing in three broiler slaughterhouses during the consecutive slaughter of broiler batches with a different *Campylobacter* status were described. As expected, slaughter of *Campylobacter* colonized batches produced contaminated carcasses. However, during consecutive slaughter of *Campylobacter* positive batches, carcass contamination in respect of the level of contamination assessed by enumeration of *Campylobacter* on breast skin remained stable. Carcasses from *Campylobacter* negative batches of broiler flocks can become contaminated with *Campylobacter* (i.e. showing counts  $\geq 10$  cfu/g) when they are processed immediately after slaughter of *Campylobacter* colonized birds. Although the number of *Campylobacter* cells transferred from positive to negative batches decreased over the first 20 minutes sampling time, the reduction was slower than it was previously estimated in risk assessment studies. Additionally, it was observed that the *Campylobacter* counts on carcasses from negative batches were influenced by the colonization level of previously slaughtered broilers and that the evisceration process was the dominant process step contributing to cross-contamination between *Campylobacter* positive and *Campylobacter* free broilers slaughtered subsequently.

## 1. Introduction

*Campylobacter* is considered to be an important cause of bacterial zoonotic infections in humans worldwide (WHO, 2012). In 2012 in the European Union, the number of campylobacteriosis cases in humans exceeded 214.000 (EFSA, 2015), but the true infection rate for all EU members might be even 46 times higher (Havelaar et al., 2012). The *Campylobacter* reservoir are warm-blooded animals, including broilers which can be colonized by more than  $10^8$  *Campylobacter* per gram in their caeca at the end of the rearing period (Ingrid Hansson et al., 2010).

The slaughter process of *Campylobacter* positive batches results not only in contamination of carcasses but also of the slaughterhouse environment (Johnsen et al., 2007; Newell et al., 2001). This implies that during the subsequent slaughter of broiler batches transmission of *Campylobacter* can occur, and as such *Campylobacter* carcass contamination can be influenced by the *Campylobacter* status of previously processed batch.

Cross-contamination is of particular concern if *Campylobacter* negative batches are processed immediately after colonized batches. To avoid contamination of negative carcasses via the slaughterhouse environment, logistic slaughter (i.e. the slaughtering of *Campylobacter* positive batches at the end of the day, after negative tested batches) has been proposed (EFSA, 2011a). However, based on quantitative risk assessment models, it has been estimated that the public health benefit of logistic slaughter is low (Evers, 2004; Nauta et al., 2005). In addition, observational data showed a limited transmission of the pathogen from a positive to a subsequent negative batch when analyzing carcass contamination after chilling (Johannessen et al., 2007). However, a more recent study (Elvers et al. 2011) indicated that carcasses from a *Campylobacter* negative batch processed immediately after a *Campylobacter* positive batch can be contaminated with numbers similar to those that can be found on carcasses from a positive batch.

This implies that the significance of cross-contamination from *Campylobacter* positive to *Campylobacter* negative batches needs to be reevaluated with regard to possible variability between batches or between slaughterhouses in the extent of transmission of (elevated) *Campylobacter* counts. It is also important to identify those steps during slaughtering that contribute most in cross-contamination with regard to the actual numbers of *Campylobacter* being transferred between batches.

Moreover, the dynamics of *Campylobacter* transfer, i.e the reduction or increase in numbers on first carcasses slaughtered either from positive batches processed after negative ones or in case of the consecutive slaughter of *Campylobacter* positive batches have not been thoroughly investigated so far.

The aim of the present study was to obtain quantitative data describing dynamics of *Campylobacter* counts on carcasses after selected processing stages in three broiler slaughterhouses during the consecutive slaughter of broiler batches with a different *Campylobacter* status.

## **2. Material and methods**

### **2.1 Samples collection**

The study was conducted in three Belgian slaughterhouses, coded A, B and C. Slaughterhouses A and B were visited five times each, whereas three visits were performed in slaughterhouse C due to the logistic reasons.

During every visit, 6 intestinal packages from each of the first four batches of the process day were collected directly after evisceration and placed in sterile plastic bags. Additionally, from the second, third and fourth batch, two carcasses were removed from the line directly after four slaughter operation steps (scalding, plucking, evisceration and washing) at 1, 10 and 20 min after the start of the slaughter of each batch and placed in the sterile plastic bags. In slaughterhouse B, carcasses were collected after bleeding instead of after scalding due to practical limitations. During the slaughterhouse visit, samples were stored at 8 °C and next, all samples were transported to the laboratory under cooled conditions. Caecal content samples were analyzed on the same day and the carcass samples stored in the laboratory overnight at 3 °C ( $\pm 2$  °C).

### **2.2 Sample preparation**

From each of the collected intestinal packages, one caecum was immersed in ethanol and, after evaporation of the ethanol, approximately 1 g of content was collected in a plastic bag. Caeca contents from each of the first batches were analyzed as a pooled sample. From all collected carcasses, approximately 10 g of breast skin was sampled (Baré et al., 2013). Therefore, for carcasses after scalding (after bleeding for slaughterhouse B), feathers were aseptically removed under sterile conditions and only breast skin was analyzed.

### **2.3 Evaluation of *Campylobacter* status of sampled batches**

The *Campylobacter* status of the sampled batches was evaluated by spreading caecal content on modified Cefaperazone Charcoal Desoxycholate Agar (mCCDA; Oxoid, England). Plates were incubated under microaerobic conditions at 41.5°C for 24 hours and after the incubation

presumptive *Campylobacter* colonies were confirmed by microscopic observation. Based on the results obtained, it was decided from which batches carcass samples were further analyzed.

## **2.4 *Campylobacter* enumeration**

All prepared samples were homogenized with 0.1% peptone water (Bio-Rad Laboratories, USA) at a ratio of 1:10, plated on Campyfood Agar (CFA; bioMérieux, France) and incubated under microaerobic conditions at 41.5°C for 48h. After incubation, colonies with typical *Campylobacter* morphology were counted and at least four presumptive *Campylobacter* colonies per sample were confirmed by microscopic examination and PCR assay (Vandamme et al., 1997).

## **2.5 Data analysis**

The *Campylobacter* counts were log<sub>10</sub>-transformed. The limit of enumeration was equal to 10 cfu/g for breast skin samples and 100 cfu/g for caecal content samples. For samples which were below the enumeration limit, the counts were set to one-half of the enumeration threshold (Rosenquist et al., 2006). Statistical analyzes were conducted using SPSS software version 22 (IBM Corporation, USA), with a significance level of 5% applied. The analyzes were performed separately for each of three evaluated scenarios: (i) when *Campylobacter* positive batches were slaughtered subsequently (scenario I), (ii) when a *Campylobacter* positive batch was slaughtered after a negative one (scenario II) and (iii) when a negative batch was slaughtered after a positive one (scenario III).

### **2.5.1 Processing of subsequent *Campylobacter* positive batches (scenario I)**

To analyze if *Campylobacter* counts differ between slaughterhouses, a general linear model with batch nested under slaughterhouse and time as a crossed factor was applied for each of the following sampling sites: after plucking, after evisceration, after washing and caeca. Differences in *Campylobacter* counts between sampling sites and between batches were analyzed per slaughterhouse using a general linear model with sampling sites and batch as crossed factors and time point nested within a batch. The analysis was followed by Tukey test to reveal significant differences in multiple comparisons.

### **2.5.2 Processing of a *Campylobacter* negative batch before a positive one (scenario II)**

Per slaughterhouse, a general linear model with time point and sampling site as two fixed factors was performed to test if *Campylobacter* counts on carcasses increase over time during the slaughter of *Campylobacter* positive birds. This was followed by Tukey test to reveal significant differences in multiple comparisons.

### 2.5.3 Processing of a *Campylobacter* positive batch before a negative one (scenario III)

If *Campylobacter* counts on carcasses from a preceding positive batch were available (slaughterhouse A - visit 3 and 5, slaughterhouse B - all visits in and slaughterhouse C - visit 1), a general linear model was applied to compare *Campylobacter* counts between positive and subsequently processed negative batch at each sampling site separately with observations grouped per visit and including time point as a crossed factor. To identify the sampling site with the highest and lowest *Campylobacter* counts transmitted from a *Campylobacter* positive to a subsequently processed negative batch, a general linear model was applied with sampling site as a fixed factor, batch as a random factor and time point nested within the batch. *Campylobacter* counts on carcasses from negative batches were compared between time points and slaughterhouses per sampling site (i.e. after plucking, after evisceration and after washing), using a general linear model with time point and slaughterhouse identified as two crossed fixed factors and visit as a random factor nested within a slaughterhouse.

## 3. Results

An overview of the *Campylobacter* counts retrieved from caecal content analyses of the first four processed batches during the day, stratified per slaughterhouse and visit, is summarized in Table 1. Collected data allowed us to evaluate *Campylobacter* counts on broiler carcasses (i) when subsequent *Campylobacter* positive batches were slaughtered (scenario I), (ii) when a *Campylobacter* positive batch was slaughtered after a negative batch (scenario II) and (iii) when a negative batch was slaughtered after a positive one (scenario III). If multiple scenarios were occurring, only one was selected (with the priority to scenario III) due to logistic limitations.

### 3.1 *Campylobacter* carcasses contamination during slaughter of successive *Campylobacter* positive batches (scenario I)

During the first visit in slaughterhouse A and slaughterhouse B, three and two successively slaughtered batches tested positive for *Campylobacter*, respectively. Caecal colonization level of broilers from these batches ranged on average between 8.40 and 8.89 log<sub>10</sub> cfu/g (Table 5.1), presenting no significant differences between batches within each slaughterhouse ( $P > 0.05$ ) and between slaughterhouses ( $P > 0.05$ ).

**Table 5.1. *Campylobacter* counts in the caecal content of first four processed batches stratified per slaughterhouse and visit. Bolded figures indicate batches from which carcass contamination was further evaluated.**

		Mean <i>Campylobacter</i> counts (SD) in caecal content (log <sub>10</sub> cfu/g)				scenario
		batch 1 <sup>a</sup>	batch 2	batch 3	batch 4	
Slaughterhouse A						
visit 1	no data	<b>8.73 (0.12)</b>	<b>8.73 (0.44)</b>	<b>8.89 (0.29)</b>	I	
visit 2	< EL	< EL	<b>8.32 (0.48)</b>	no data	II	
visit 3	no data	9.48 (0.16)	<b>8.90 (1.03)</b>	< EL	III	
visit 4	<b>8.73</b>	< EL	< EL	7.51 (0.99)	III	
visit 5	6.04	<b>5.62 (0.89)</b>	< EL	8.95 (0.53)	III	
Slaughterhouse B						
visit 1	< EL	<b>8.40 (0.63)</b>	<b>8.67 (0.30)</b>	< EL	I	
visit 2	< EL	< EL	<b>7.28 (2.75)</b>	8.11 (0.51)	II	
visit 3	< EL	3.94 (3.04)	<b>9.12 (0.69)</b>	< EL	III	
visit 4	< EL	<b>8.39 (0.34)</b>	< EL	< EL	III	
visit 5	< EL	8.40 (0.63)	<b>8.67 (0.30)</b>	< EL	III	
Slaughterhouse C						
visit 1	8.98	<b>8.83 (0.86)</b>	< EL	no data	III	
visit 2	<b>7.90</b>	< EL	< EL	< EL	III	
visit 3	<b>9.82</b>	< EL	8.99 (0.34)	8.80 (0.34)	III	

< EL – less than enumeration limit = 100 cfu/g

<sup>a</sup> caeca contents from each of the first batches were analyzed as a pooled sample

I – subsequent *Campylobacter* positive batches were slaughtered

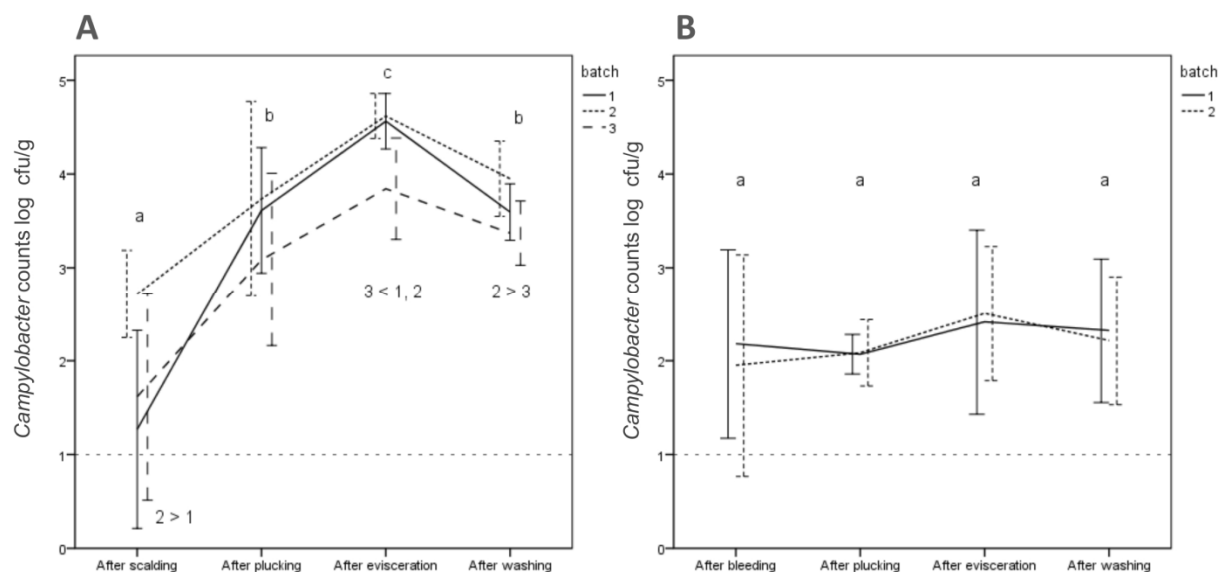
II - *Campylobacter* positive batch slaughtered after a negative batch

III – *Campylobacter* negative batch slaughtered after a positive batch

Analysis of carcass contamination showed that *Campylobacter* counts after plucking, evisceration and washing were significantly lower in slaughterhouse B than in slaughterhouse A ( $P < 0.001$ ).

During processing of *Campylobacter* positive batches in slaughterhouse A (Fig. 5.1.A), *Campylobacter* counts were significantly lower after scalding ( $P < 0.001$ ) and significantly higher after evisceration ( $P < 0.05$ ) in comparison to the three other sampling sites. Additionally, variability in *Campylobacter* counts between batches was observed in slaughterhouse A. Carcasses from batch two carried significantly more *Campylobacter* after scalding and after washing in comparison to batch 1 and 3, respectively ( $P < 0.05$ ). After evisceration, *Campylobacter* counts on carcasses from the third batch were significantly lower contaminated than those on carcasses from the other two batches ( $P < 0.05$ ).

In contrast, in slaughterhouse B (Fig. 5.1.B), no significant differences in *Campylobacter* counts between sampling sites as well as between batches were detected ( $P > 0.05$ ).



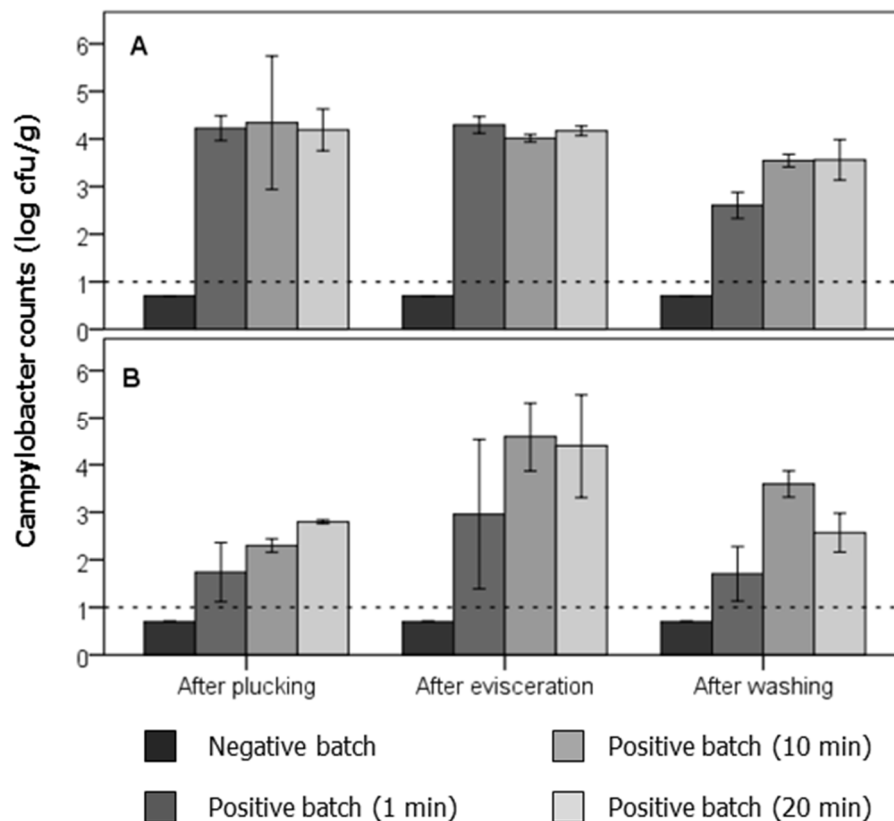
**Figure 5.1.** *Campylobacter* counts (mean  $\log_{10}$  cfu/g  $\pm$  SD) on carcass breast skin collected after scalding (after bleeding in slaughterhouse B), plucking, evisceration and washing in two slaughterhouses (A and B) during the subsequent slaughter of *Campylobacter* positive batches. The horizontal dashed line indicates the enumeration limit (10 cfu/g breast skin). Per slaughterhouse, different letters above the lines define significant differences in *Campylobacter* counts between sampling sites. Numbers below the lines indicate significant differences in *Campylobacter* counts between batches per each sampling site. Lack of this information expresses no significant differences between batches at the certain sampling site.

### 3.2 *Campylobacter* contamination of carcasses from a negative and a following positive batch (situation II)

During the second visit in slaughterhouse A and slaughterhouse B, *Campylobacter* was not detected in the caecal content of birds from the first two batches. In contrast, broilers from the third batch were colonized on average with more than 7  $\log_{10}$  cfu/g (Table 5.1).

As presented in Figure 5.2, no *Campylobacter* was recovered on carcasses when *Campylobacter* negative birds were slaughtered as the first batch during the processing day. However, as soon as birds from a *Campylobacter* positive batch entered the slaughter line, *Campylobacter* carcass contamination with numbers  $\geq 10$  cfu/g occurred. In slaughterhouse A, an immediate increase of carcass contamination (up to approximately 4  $\log_{10}$  cfu/g) was observed already after the first minute of slaughter and the contamination level remain stable over 20 minutes of slaughter process. In contrast in slaughterhouse B, *Campylobacter* counts on carcasses from the first minute were significantly lower ( $P < 0.05$ ) than on carcasses collected after 10 and 20 min from the start of a *Campylobacter* positive batch.





**Figure 5.2.** *Campylobacter* counts (mean log<sub>10</sub> cfu/g ± SD) on carcass breast skin collected in slaughterhouses (A, B) after plucking, evisceration and washing. Black bars represent samples (n = 6) collected from a *Campylobacter* negative batch processed directly before a positive one. Gray bars represent samples (n = 2) collected at 1, 10 and 20 min from the start of the slaughter of a *Campylobacter* positive batch. The horizontal dashed line indicates the enumeration limit (10 cfu/g breast skin).

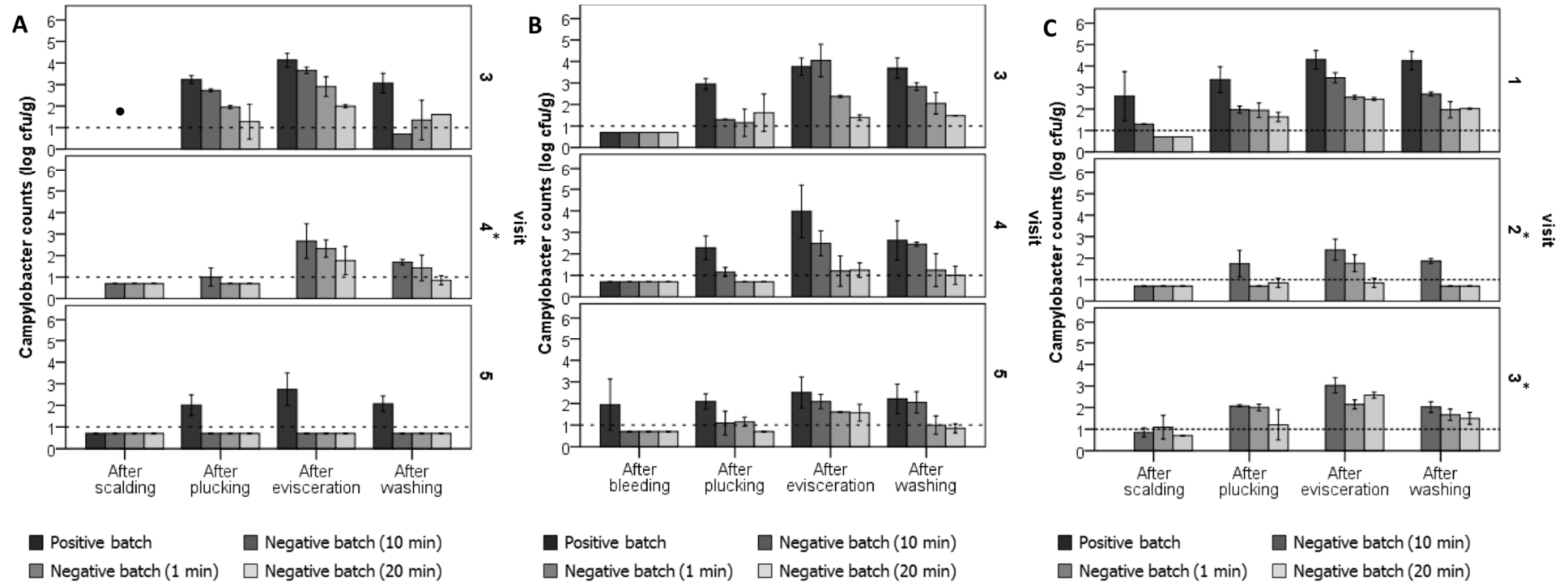
### 3.3 *Campylobacter* contamination of carcasses from negative batches slaughtered after positive batches (situation III)

During three visits in each of three slaughterhouses (visits 3, 4 and 5 in slaughterhouses A and B and visits 1, 2 and 3 in slaughterhouse C; Table 5.1), *Campylobacter* was enumerated on carcasses from *Campylobacter* negative batches processed after positive ones. *Campylobacter* caecal colonization level of birds from proceeding *Campylobacter* positive batches ranged on average between 5.62 and 9.82 log<sub>10</sub> cfu/g. The slaughter of these birds caused an (enumerable) *Campylobacter* carcass contamination along the process line with average counts equal to 3.22, 4.06 and 3.68 log<sub>10</sub> cfu/g on carcasses collected after plucking, evisceration and washing, respectively (Fig. 5.3). Additionally, slaughter of positive birds contributed to the *Campylobacter* contamination (in numbers ≥ 10 cfu/g) of broiler carcasses from subsequently processed negative batches (Fig. 5.3). However, obtained quantitative results showed that *Campylobacter* counts on samples collected during the slaughter of negative batch were significantly lower in comparison to those from prior processed positive batches ( $P < 0.01$ ; Fig. 5.3). On average

carcasses from these negative batches were contaminated with *Campylobacter* at the level of 1.53, 2.73 and 1.89 log<sub>10</sub> cfu/g on carcasses collected after plucking, evisceration and washing, respectively. Moreover, the transmission rates varied between sampling sites, with significantly the lowest and the highest ( $P < 0.01$ ) *Campylobacter* counts after scalding and after evisceration, respectively (Fig. 5.3).

Quantitative analyzes of samples collected at 1, 10, and 20 min from the start of a negative batch revealed a decreasing trend in *Campylobacter* counts over time when analyzing carcasses after plucking, evisceration and washing. At these sampling sites, *Campylobacter* counts were significantly higher ( $P < 0.01$ ) on carcasses collected during the first minute in comparison to those from the last time point of sampling (20 minutes; Fig. 5.3).

Interestingly, when birds with the low colonization level were slaughtered (visit 5 in slaughterhouse A), *Campylobacter* was not detected in numbers exceeding 10 cfu/g on carcasses collected from a subsequently processed negative batch (Fig 5.3.A).



**Figure 5.3. *Campylobacter* counts (mean log<sub>10</sub> cfu/g ± SD) on carcass breast skin collected in three slaughterhouses (A, B and C) after scalding (after bleeding in slaughterhouse B), plucking, evisceration and washing. Black bars represent samples (n = 6) collected from a positive batch processed directly before a negative one. Gray bars represent samples (n = 2) collected at 1, 10 and 20 min from the start of the slaughter of a *Campylobacter* negative batch. The horizontal dashed line indicates the enumeration limit (10 cfu/g breast skin). Bullet indicates location, from which sample were not collected during the first visit in slaughterhouse A. Asterisk indicates visits, from which carcass samples from a preceding *Campylobacter* positive batch were not collected.**

## 4. Discussion

In the present study, *Campylobacter* counts on broiler carcasses from successively slaughtered batches with different *Campylobacter* status have been described. *Campylobacter* was not detected in the present study on carcasses from *Campylobacter* negative batches processed at the beginning of the working day. Also in the Norwegian study (Johannessen et al., 2007), when the direct plating method was applied, *Campylobacter* was not detected on carcasses from the first batch during the day when caecal samples were negative. This indicates good cleaning and disinfection practices in the visited slaughterhouses prior to startup of the slaughtering process what prevents *Campylobacter* from the enumerable day-to-day transmission.

As it has been previously reported (Allen et al., 2007; Duffy et al., 2014; Sampers et al., 2010; Seliwiorstow et al., 2015), slaughtering of positive broilers resulted in *Campylobacter* carcass contamination across the slaughter line. Although, in the present study, we also observed that *Campylobacter* counts on positive carcasses remain stable if only positive batches (with similar level of caecal colonization) were slaughtered. This might indicate that the main source of carcass contamination is a positive flock itself and that the contamination of the slaughterhouse environment does not play a significant role when *Campylobacter* positive batches are slaughtered subsequently.

It has been shown that a contaminated slaughterhouse environment is a vector for *Campylobacter* transmission from positive to *Campylobacter* free broilers (Corry and Atabay, 2001; Guerin et al., 2010). However, the latter studies used presence-absence testing of *Campylobacter*, for assessing its transmission, whereas nowadays relationship between *Campylobacter* counts and consumers' health risk is established (Callicott et al., 2008; Nauta and Havelaar, 2008). Therefore, in the present study, *Campylobacter* cross contamination, between positive and successively slaughtered negative batches, was quantitatively assessed after four process stages: scalding/bleeding, plucking, evisceration and washing. The lowest numbers of *Campylobacter* were transmitted between batches during scalding. This observation can be explained by the temperature of scalding water (Yang et al., 2001) and additionally by a simple dilution effect. Carcasses were scalded at 52 - 53, 54 and 50.5 - 52.5 °C for at least 145 seconds in slaughterhouses A, B and C, respectively. In contrast to scalding, the highest *Campylobacter* counts were observed on carcasses collected after evisceration, which might be justified by the frequent rupture of intestines during evisceration. In slaughterhouses A, B and C fifteen, seven and forty four percent of intestines were ruptured after the evisceration process, respectively. Leaking intestinal content significantly increases contamination of carcasses (Berrang et al., 2004; Boysen and Rosenquist, 2009; Smith et al., 2007) and also potentially evisceration equipment which is further a significant route of contamination for carcasses from subsequently slaughtered negative batches (Posch et al., 2006).

In addition, results obtained in the present study showed that a decrease of *Campylobacter* counts in caecal content would not only reduce the carcass contamination when *Campylobacter* positive batches are slaughtered (Reich et al., 2008; Rosenquist et al., 2006), but thus would also limit cross-contamination of subsequently processed *Campylobacter* negative broilers.

To avoid cross-contamination from the *Campylobacter* positive to negative batches, logistic slaughter (i.e. procedure where positive batches are processed after negative batches) has been suggested as a possible intervention strategy (EFSA, 2011a). However, risk assessment studies presented a limited effect of logistic slaughter on human health risk reduction (Havelaar et al., 2007; Nauta et al., 2005; Rosenquist et al., 2003). In the present study, *Campylobacter* counts were lower on carcasses from negative batches in comparison to those from positive ones and additionally they declined over time when negative batches were processed. Thus the trend in the present study agrees with the observation of *Campylobacter* cross-contamination between positive and negative batches of broiler flocks Elvers et al. (2011) and Johannessen et al. (2007). However, the generalized concept that a limited number of carcasses from a negative batch became contaminated by *Campylobacter* originating from preceding positive broilers (EFSA, 2011a; Johannessen et al., 2007; Nauta et al., 2009) is questioned. In present study, during twenty minutes of operation approximately 3,500, 4,000 and 2,000 carcasses (from the consecutive *Campylobacter* negative batch) can pass the slaughter line in slaughterhouses A, B and C, respectively. At this moment, *Campylobacter* was still enumerated at the highest (among three visits) average level equal to 1.60, 1.48 and 2.02 log<sub>10</sub> cfu/g on carcasses after washing for slaughterhouse A, B and C, respectively. In general in the present study 18 analyzed carcasses collected after washing at 20 minutes from the start of negative batch 11 of them were contaminated with *Campylobacter* at the level higher than 10 cfu/g. In contrast, it was estimated than only approximately first 20 carcasses in a negative batch processed after a positive batch will be contaminated with *Campylobacter* (Nauta et al., 2005).

As assumed in risk assessment studies, combination of low contamination removal by passing carcasses (low line speed) (Nauta et al., 2005; Rosenquist et al., 2003) and high environmental contamination (e.g. high fraction of broken intestines) should result in higher *Campylobacter* counts transferred from positive to negative batches in slaughterhouse C, which holds a lower slaughter speed (6.000 birds/hour) compared to the others (11.000 and 12.000 birds/hour for slaughterhouse A and B, respectively) Further, intestines from birds slaughtered at slaughterhouse C are more frequently ruptured (44%) in comparison with slaughterhouse A (15%) or B (7%). However, based on the results obtained, significant difference between slaughterhouses in the extent of *Campylobacter* transmission was not observed, suggesting that other, at present unknown, factors might influence *Campylobacter* cross-contamination between batches.

In conclusion, the present study confirmed that the status of slaughtered birds determines carcass contamination. Slaughter of *Campylobacter* colonized batches produces contaminated carcasses, although if only *Campylobacter* positive batches are processed subsequently *Campylobacter* carcass contamination remains stable throughout the working day. Non-contaminated carcasses are produced if only *Campylobacter* negative batches are slaughtered. However, carcasses from *Campylobacter* negative batches can become contaminated when they are processed immediately after *Campylobacter* colonized birds. This cross-contamination decreases over time but slower than it was previously estimated. Additionally, it was noted that *Campylobacter* counts on carcasses from negative batches are influenced by the colonization level of previously slaughtered broilers and that the evisceration process contribute the most to the cross-contamination between positive and *Campylobacter* free batches. Therefore, intervention aiming at the reduction of *Campylobacter* counts in caecal content or additional treatment of evisceration machine between batches could decrease the extent of *Campylobacter* transmission from a positive to a subsequently processed negative batch.

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**IDENTIFICATION OF RISK FACTORS ASSOCIATED WITH  
ENUMERABLE *CAMPYLOBACTER* CARCASS  
CONTAMINATION IN BROILER SLAUGHTERHOUSES.**

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## Abstract

The aim of the present study was to collect data on *Campylobacter* broiler carcass contamination ( $\geq 10$  cfu/g) across the slaughter line and to identify factors associated with *Campylobacter* counts. Our results confirmed the importance of the initial external carcass contamination at the beginning of the slaughter line. As expected it was shown that the plucking, evisceration, washing and chilling step can influence *Campylobacter* counts on broiler carcasses. As such at each of these locations (i.e. after bleeding – initial contamination, after plucking, after evisceration, after washing and after chilling), factors associated with *Campylobacter* counts on slaughtered carcasses were studied. The results obtained indicated that reduction of *Campylobacter* colonization level and optimization of transport and holding time might result in a lower broiler carcass contamination across the slaughter line. Additionally, dump based unloading system, electrical stunning, too low scalding temperature incorrect setting of plucking, vent cutter and evisceration machines were identified as risk factors associated with an increase of *Campylobacter* counts on broiler carcasses. Importantly, all investigated factors were existing variations of the routine processing practices and therefore proposed interventions can be practical and economical achievable.

## 1. Introduction

Campylobacteriosis remains the most frequently reported zoonotic disease in humans in the European Union (EU) with reported number of cases exceeding 214,000 in 2013 (EFSA, 2015). However, it is estimated that annually more than 9 million people in the EU can suffer from campylobacteriosis, with diarrhea, abdominal pain, fever, headache and nausea as common symptoms (Havelaar et al., 2012). In sporadic cases, severe post-infectious complications such as Guillian–Barré syndrome (Nachamkin, 2002), reactive arthritis (Hannu et al., 2002), inflammatory bowel disease (Man, 2011) or even death may occur (EFSA, 2015).

Multiple sources for *Campylobacter* infection have been identified (Kittl et al., 2013; Lévesque et al., 2013), but 20 to 30% of campylobacteriosis cases in humans are attributed to the consumption and handling of broiler meat (EFSA, 2011). Therefore, there is a need for applicable interventions in the broiler meat sector aiming to reduce human exposure to *Campylobacter*. Over seventy per cent of the slaughtered broiler batches in Europe are colonized with *Campylobacter* (EFSA, 2010). Lowering the prevalence or the colonization level at the primary production would considerably decrease the consumer risk of campylobacteriosis through poultry meat consumption (Havelaar et al., 2007). However, no effective measures to prevent or reduce *Campylobacter* colonization in broilers are currently available (Hermans et al., 2011; Lin, 2009; Newell et al., 2011).

Slaughter of *Campylobacter* positive broilers results in carcasses contaminated with high (up to 6 log<sub>10</sub> cfu/carcass or 4 log<sub>10</sub> cfu/g) *Campylobacter* numbers (Allen et al., 2007; Duffy et al., 2014; Reich et al., 2008; Rosenquist et al., 2006; Seliwiorstow et al., 2015). To reduce *Campylobacter* counts on poultry meat, several interventions including freezing (Tustin et al., 2011), crust freezing (Haughton et al., 2012), ultraviolet light (Haughton et al., 2011), irradiation (Kudra et al., 2012) and chemical decontamination (Riedel et al., 2009) have been proposed or applied. Nevertheless, consumer acceptability (MacRitchie et al., 2014) as well as economic (Havelaar et al., 2007) and legal (Hugas and Tsigarida, 2008) arguments limit the worldwide implementation of these practices.

Alternatively to physical or chemical treatments, optimization of the technical and hygiene-related factors might reduce *Campylobacter* counts on broiler carcasses (Habib et al., 2012; Wagenaar et al., 2013). *Campylobacter* is not able to multiply outside the host what implies that high counts of bacteria are needed to reach the consumer via cross-contamination (Kusumaningrum et al., 2004). Therefore, a reduction in human campylobacteriosis cases does not require *Campylobacter*-free carcasses (Callicott et al., 2008; Nauta et al., 2009). Already compliance with the limit of 1 000 or 500 cfu/g on the neck/breast skin can reduce the number human campylobacteriosis cases by more than 50 or 90%, respectively (EFSA, 2011).

To enable slaughterhouses to meet these criteria, factors that influence *Campylobacter* carcass contamination need to be identified. Therefore, a risk factor analyses was performed on *Campylobacter* data obtained across the slaughter line in six Belgian slaughterhouses.

## **2. Materials and methods**

### **2.1. Selection of slaughterhouses**

Six Belgian broiler slaughterhouses with a minimum annual production capacity of 10 million broilers each were included in the study and coded from A to F. Based on data obtained during the 2008 EFSA baseline study two of them (slaughterhouses B and E) had higher *Campylobacter* prevalence on chilled carcasses in comparison to slaughterhouses C and D (Habib et al., 2012). Slaughterhouse A and F were not included during the 2008 EFSA baseline study. Each slaughterhouse was certified by a third party for operating under at least one voluntary quality management system. They were all equipped with the automated processing line, with slaughter capacity range between 6 and 12 thousands birds per hour.

### **2.2. Slaughterhouse and batch related process characteristics**

To collect information about the technical aspects of the slaughter process, two questionnaires were designed, based on literature review. The questionnaires consisted of a series of questions related to factors potentially associated with *Campylobacter* counts on broiler carcasses. The first questionnaire was completed by the researcher, before sampling, during an interview with the slaughterhouses' representative and the initial slaughter line inspection. This questionnaire focused on general management and technical slaughter line characteristics. Information obtained by this questionnaire resulted in the identification of 20 slaughterhouse related explanatory variables (Table 6.1). The second questionnaire focused on batch related processing conditions and was completed during every sampling visit based on the researcher observations and the slaughterhouse records. Information obtained by this questionnaire resulted in the identification of 9 batch related explanatory variables (Table 6.1).

**Table 1. Slaughterhouse and batch related explanatory variables used for the risk factors identification.**

No.	Explanatory variable	Modality
<b>Slaughterhouse (slh) related explanatory variable</b>		
1	Line speed (thousand carcasses/hour)	Slh. A: 11, B: 12.7, C: 9, D: 12, E: 10.5, F: 6
2	separate lines per broilers age (size) or applied size criteria for processed carcasses	0 - No (slh. B, F) 1 - Yes (slh. A, C, D, E)
3	Stunning	0 - Gas (slh. D, B - visit 4, 5) 1 - Electrical (slh A, B – visit 1 to 3, C, E, F)
4	Unloading system	0 - drawers (slh. A) 1 - containers (slh. B, C, D, E, F)
5	Counter flow of clean water in scalding tanks	0 - No (slh. A, C) 1 - Yes (slh. B, D, E, F)
6	Scalding time (seconds)	Slh. A: 149, B: 252, C: 138, D: 140, E: 255, F: 210
7	Age of plucking equipment (years)	Slh. A: 5, B: 14, C: 14, D: 18, E: 6, F: 25
8	Number of plucking machines	Slh. A: 1, B: 6, C: 3, D: 5, E: 3, F: 1
9	Plucking time (seconds)	Slh. A: 33, B: 47, C: 44, D: 43, E: 30, F: 60
10	Age of evisceration equipment (years)	Slh. A: 5, B: 5, C: 14, D: 7, E: 6, F: 17
11	Adjustment of evisceration machine (between processed batches)	0 - No (slh. C, F) 1 - Yes (slh. A, B, D, E)
12	Number of working nozzles spraying carcass with water after evisceration	Slh. A: 1, B: 2, C: 4, D: 1, E: 30, F: 2
13	Working brush cleaning drill at the crop puller equipment	0 - No (slh. D, F) 1 - Yes (slh. A, B, C, E)
14	Number of nozzles spraying carcass with water after crop puller	Slh. A: 2, B: 0, C: 2, D: 2, E: 0, F: 0
15	Final outside-inside washer present	0 - No (slh. C) 1 - Yes (slh. A, B, D, E, F)
16	Water in chiller sprayed on carcasses	0 - No (slh. A, D, E) 1 - Yes (slh. B, C, F)
17	Multilevel chiller <sup>a</sup>	0 - No (slh. A, B, C, F) 1 - Yes (slh. D, E)
18	Air temperature in chillers (°C)	Slh. A: 2, B: 3, C: 0, D: -2, E -2, F: 1.5

19	Maximum chilled internal carcass temperature (°C)	Slh. A: 4, B: 11, C: 5, D: 3, E 4, F: 6
20	Chilling time (min)	Slh. A: 105, B: 120, C: 135, D: 180, E: 105, F: 120

#### Batch related explanatory variable

21	Transport and holding time	[3.5 – 14 h]
22	Temperature of scalding water <sup>b</sup>	[50.5 – 55.0 °C]
23	Visual fecal contamination level of the floors within the transporting crates	1 - low, 2 -medium, 3 - high, 4 - very high
24	Visual fecal contamination level of carcasses at hanging area	1 - low, 2 -medium, 3 - high, 4 - very high
25	Percentage of carcasses with feathers on breast after plucking	[0 – 37.7 %]
26	Percentage of carcasses with damaged cloaca	[0 – 8.0 %]
27	Percentage of carcasses without total losing of cloaca	[0.6 – 49.3 %]
28	Percentage of ruptured gastrointestinal packages	[0.5 – 42.0 %]
29	Average carcass weight (n =6, measured after chilling)	[1578 – 2155 g]

#### Batch related bacteriological data

30	<i>Campylobacter</i> in scalding water <sup>c</sup>	[0.7 <sup>f</sup> – 2.97 log <sub>10</sub> cfu/ml]
31	Mean <i>Campylobacter</i> counts in caecal content (n=6)	[6.68 – 9.71 log <sub>10</sub> cfu/g]
32	Mean <i>Campylobacter</i> counts in duodenal content (n=6)	[1.57 – 6.26 log <sub>10</sub> cfu/g]
33	Mean <i>Campylobacter</i> counts on feather samples <sup>d</sup> (n=6)	[0.7 <sup>f</sup> – 6.81 log <sub>10</sub> cfu/g]
34	<i>Campylobacter</i> status of previous batch	1 – positive 0 – negative/no previous batch
35	Mean <i>Campylobacter</i> counts in caecal content of previous batch (pooled sample from ten collected caecal contents)	[1.7 <sup>f</sup> – 9.40 log <sub>10</sub> cfu/g]
36	Mean <i>Campylobacter</i> counts in duodenal content of previous batch (pooled sample from ten collected duodenal contents)	[1.7 <sup>f</sup> – 6.51 log <sub>10</sub> cfu/g]
37	<i>Campylobacter</i> in chilling water <sup>c</sup>	[3.01 – 3.37 log <sub>10</sub> cfu/ml]
38	Mean <i>Campylobacter</i> counts on carcasses collected after bleeding	[0.7 <sup>f</sup> – 5.29 log <sub>10</sub> cfu/g]
39	Mean <i>Campylobacter</i> counts on carcasses collected after plucking	[2.33 – 4.13 log <sub>10</sub> cfu/g]
40	Mean <i>Campylobacter</i> counts on carcasses collected before washing	[2.16 – 4.27 log <sub>10</sub> cfu/g]
41	Mean <i>Campylobacter</i> counts on carcasses collected after washing	[2.03 – 4.05 log <sub>10</sub> cfu/g]

<sup>a</sup>Carcasses were chilled at multiple levels. The most chilled carcasses were at the bottom. Water can drip from less chilled carcasses above to more chilled carcasses on the lower levels.

<sup>b</sup>Average temperature of measurements at the beginning and the end of every scalding tank.

<sup>c</sup>Mean *Campylobacter* counts in scalding/chilling water samples collected at the beginning and at the end of every scalding/chilling tank.

<sup>d</sup>From each of collected carcass after bleeding *ca.* 10g of feathers were removed manually and analyzed separately.

<sup>f</sup>Half of the detection limit

### 2.3. Collection of batch related bacteriological data.

For each followed batch six feather samples (from carcasses collected after bleeding), six caecal and duodenal samples (collected after the evisceration process) were analyzed. Additionally, scalding water samples were collected from the beginning and the end of every scalding tank. Likewise, chilling water was also sampled in slaughterhouse C. If the sampled batch was not the first during the processing day, pooled caecal ( $n = 10$ ) and duodenal ( $n = 10$ ) samples were examined from the preceding slaughtered batch. For all sample types, *Campylobacter* enumeration was performed as described in Chapter 3.

### 2.4. Collection of *Campylobacter* quantitative data on carcass contamination

Previously collected data (Chapter 3) describing *Campylobacter* carcass contamination along the processing line in four slaughterhouses (A - D) during slaughter of three *Campylobacter* positive batches (i.e. birds from one flock slaughtered at the same day) in each slaughterhouse were used in the present study. Additionally, two other *Campylobacter* positive batches were sampled in each of slaughterhouses A - D, except for slaughterhouse C which stopped its production activity during the study. Furthermore, in each of slaughterhouses E and F five *Campylobacter* positive batches were sampled. Altogether, *Campylobacter* carcass contamination was assessed by using enumeration methods during the slaughter of 28 *Campylobacter* positive broiler batches during the period of February 2011 to November 2013. Identification of *Campylobacter* positive batches on farms, sampling strategy at the slaughterhouse and *Campylobacter* enumeration was performed as described in Chapter 3.

### 2.5. Statistical analysis

*Campylobacter* counts on carcasses were compared between slaughterhouses, between selected sampling sites within each slaughterhouse with the same method as in Chapter 3.

Multilevel mixed-effects negative binomial model including batch as a random factor was applied to investigate the associations between *Campylobacter* counts and the potential explanatory variables at each of four selected sampling sites. A stepwise forward inclusion model-building strategy was used, including at each step the significant variable until only the non-significant variables remained. If more than one explanatory variable was significant at the certain step of the model building process, one of them was chosen based on Akaike information criterion (AIC).

Statistical analyses were carried out using commercial software (Stata/SE 13.1 StataCorp LP, College Station, TX) and a significance level of 5% was used.

### 3. Results

#### 3.1. *Campylobacter* carcass contamination during slaughter.

In total, *Campylobacter* was quantified on 1176 broiler carcasses originating from 28 *Campylobacter* positive batches sampled in six slaughterhouses along the slaughter line.

A high variability in *Campylobacter* counts within each batch and between batches was observed. In contrast, the differences between slaughterhouses were significant only at the beginning of the line (after bleeding) with significantly lower *Campylobacter* counts on carcasses collected at this sampling site in slaughterhouse A in comparison to carcasses after bleeding collected in the other slaughterhouses ( $P < 0.05$ ). No significant differences were observed between slaughterhouses for the following process steps ( $P > 0.05$ ).

When analyzing *Campylobacter* counts per slaughterhouse along the processing line, carcass contamination after plucking was significantly higher than the *Campylobacter* counts at the beginning of the slaughter line (after bleeding) in slaughterhouses A and E ( $P < 0.05$ ). In slaughterhouse A, B and D *Campylobacter* counts significantly increased after the evisceration process when comparing carcasses after evisceration to carcasses after plucking. Chilling had no significant effect on *Campylobacter* counts when comparing samples collected after washing (before chilling) and after chilling in any of visited slaughterhouses ( $P > 0.05$ ). In contrast, when comparing carcass contamination before washing and after chilling, a significant decrease in *Campylobacter* counts was observed in slaughterhouses B, D, E and F ( $P < 0.05$ ). The quantitative *Campylobacter* carcass contamination was not significantly influenced by the crop pulling process when comparing carcass contamination after crop pulling and contamination after evisceration ( $P > 0.05$ ).

#### 3.2. Risk factors identification.

Risk factors for high quantitative *Campylobacter* contamination levels on broiler carcasses collected at five selected sampling sites are presented in Table 6.2.

Obtained results revealed the significance of internal and external carriage of *Campylobacter* of incoming birds. *Campylobacter* counts on carcasses collected after plucking, evisceration, washing and chilling were influenced by the level of *Campylobacter* caecal colonization ( $P < 0.01$ ). Also quantitative contamination of feathers was associated with *Campylobacter* counts recovered from skin samples collected at the beginning of the slaughter line ( $P < 0.001$ ).

Moreover, the time that birds were crated was negatively associated with *Campylobacter* counts at all sampling sites ( $P < 0.05$ ). The longer transport and holding time, the lower *Campylobacter* counts were recovered.



**Table 6.2. Risk factors associated with changes in *Campylobacter* counts on carcasses collected after selected processing steps.**

Sampling site	Explanatory variable	Coef.	CI 95%	P - value
After bleeding	Unloading system			
	– drawers	-	-	-
	– container	1.82	[0.21, 3.42]	0.027
	Transport and holding time	- 0.35	[-0.63, -0.07]	0.013
	Mean <i>Campylobacter</i> counts on feather samples <sup>a</sup>	1.15	[0.73, 1.57]	<0.001
After plucking	Stunning			
	– gas	-	-	-
	– electrical	2.08	[1.00, 3.15]	<0.001
	Holding and transport time	- 0.31	[-0.51, -0.11]	0.002
	Mean <i>Campylobacter</i> counts in caecal content	0.85	[0.28, 1.43]	0.004
After evisceration	Transport and holding time	- 0.22	[-0.40, -0.04]	0.018
	Temperature of scalding water <sup>b</sup>	- 0.52	[-0.93, -0.12]	0.011
	Percentage of carcasses with feathers on breast after plucking	- 0.12	[-0.23, -0.01]	0.033
	Mean <i>Campylobacter</i> counts in caecal content	0.95	[0.41, 1.49]	0.001
After Washing <sup>c</sup>	Stunning			
	– gas	-	-	-
	– electrical	1.12	[0.40, 1.85]	0.002
	Transport and holding time	- 0.21	[-0.35, -0.07]	0.003
	Percentage of carcasses with damaged cloaca	0.17	[0.03, 0.31]	0.015
	Mean <i>Campylobacter</i> counts in caecal content	0.79	[0.40, 1.18]	<0.001
After chilling	Transport and holding time	- 0.29	[-0.50, -0.08]	0.006
	Percentage of ruptured gastrointestinal packages	0.06	[0.02, 0.11]	0.004
	Mean <i>Campylobacter</i> counts in caecal content	1.83	[1.22, 2.44]	<0.001

<sup>a</sup>From each of collected carcass after bleeding *ca.* 10g of feathers were removed manually and analyzed separately.

<sup>b</sup>Average temperature of measurements at the beginning and the end of every scalding tank.

<sup>c</sup>After water chilling for slaughterhouse C.

Furthermore, it was shown that some technical aspects of the slaughter process can be associated with *Campylobacter* counts on broiler carcasses. An unloading system with drawers was a protective factor for *Campylobacter* contamination at the beginning of the slaughter line (after bleeding). The average *Campylobacter* counts on breast skin samples collected from batches being unloaded with the container system were  $3.07 \pm 1.41 \log_{10} \text{ cfu/g}$  ( $n = 138$  carcasses), whereas broilers from batches unloaded with the drawers system carried on average  $1.66 \pm 1.26 \log_{10} \text{ cfu/g}$  *Campylobacter* on their breast skin ( $n = 30$ ). Not only the unloading system but also the stunning method was associated with *Campylobacter*

counts. Electrical stunning was elucidated as a risk factor for increased *Campylobacter* carcasses contamination after plucking and after washing ( $P < 0.001$ ). Broilers from seven batches (2 in slaughterhouse B and 5 in slaughterhouse D; Table 1), which were stunned by gas carried on average  $2.81 \pm 0.47$  and  $2.72 \pm 0.55 \log_{10}$  cfu/g *Campylobacter* on their breast skin after plucking ( $n = 42$  carcasses) and washing ( $n = 42$ ), respectively. In contrast, average *Campylobacter* counts on carcasses collected from batches that were stunned with electricity were equal to  $3.25 \pm 0.67$  and  $3.21 \pm 0.59 \log_{10}$  cfu/g after plucking ( $n=126$ ) and after washing ( $n=126$ ), respectively. Furthermore, the temperature of the scalding tanks was negatively associated with *Campylobacter* counts on carcasses after evisceration ( $P = 0.011$ ). Most of analyzed broilers were scalded below  $54^{\circ}\text{C}$ , only during processing of four batches in slaughterhouse D, scalding temperature was registered at the level between  $54$  and  $55^{\circ}\text{C}$ , which resulted in a lower carcass contamination. According to our findings also percentage of carcasses with feathers on breast skin after plucking was negatively associated with *Campylobacter* counts on broiler carcasses after evisceration. We observed that batches, where more than 5 % of carcasses had feathers present on the breast skin after plucking ( $n = 65$  carcasses), *Campylobacter* counts was on average  $0.4 \log_{10}$  cfu/g lower in comparison to those from better plucked batches. Moreover, percentage of ruptured gastrointestinal packages and broken cloaca were positively associated with *Campylobacter* counts on broiler carcasses collected after washing and chilling, respectively. In 7 batches (1 in slaughterhouse A, 1 in slaughterhouse C and 5 in slaughterhouse F), the percentage of ruptured gastrointestinal packages (i.e. the number of ruptured gastrointestinal packages in relation to the number of carcasses slaughtered per minute) was higher than 10 %. In such cases, mean *Campylobacter* counts on carcasses collected after chilling was equal to  $3.20 \pm 0.80 \log_{10}$  cfu/g ( $n = 42$  carcasses). In contrast, when less than 10 % of gastrointestinal packages were broken, average *Campylobacter* counts came down to  $2.81 \pm 0.55 \log_{10}$  cfu/g ( $n = 126$  carcasses). Percentage of registered broken cloaca (i.e. the number of broken cloaca in relation to the number of carcasses slaughtered per minute) after the vent cutter was lower than the percentage of ruptured gastrointestinal packages (Table 1). *Campylobacter* counts on carcasses after washing in four batches (1 in slaughterhouse C and 3 in slaughterhouse F) with more than 5 % broken cloaca were  $3.34 \pm 0.57 \log_{10}$  cfu/g ( $n = 24$  carcasses), whereas carcasses from batches with less than 5 % broken cloaca were contaminated with on average  $2.97 \pm 0.62 \log_{10}$  cfu/g ( $n = 144$  carcasses).

## 4. Discussion

The present study determined quantitative data on *Campylobacter* carcass contamination in 6 Belgian poultry slaughterhouses, showing a high variability in *Campylobacter* counts within each batch and between batches but not between slaughterhouses. This indicates that the variance in *Campylobacter*

counts obtained in the present study can be better explained by differences between batches rather than between slaughterhouses.

When comparing the different sampling sites on the slaughter line, it was observed that the change in *Campylobacter* counts during plucking and evisceration differs according to the contamination level of the carcasses at the beginning of the slaughter process. As described before (Seliwiorstow et al., 2015), during the slaughter of batches with low initial level of contamination, *Campylobacter* counts significantly increased after plucking and evisceration, whereas in batches with high contamination level after bleeding, plucking and evisceration did not significantly influence *Campylobacter* carcass contamination. Similarly to our findings Rosenquist et al. (2006) observed an increase in *Campylobacter* counts after evisceration in a slaughterhouse with (a relatively) low carcass contamination after plucking ( $< 2.8 \log \text{ cfu/g}$ ). Nevertheless, in a slaughterhouse with a higher carcass contamination after plucking ( $> 3.8 \log \text{ cfu/g}$ ), evisceration did not influence *Campylobacter* counts. Moreover, Berrang and Dickens (2000) and Elvers et al. (2011) reported even significant reductions in *Campylobacter* counts after the plucking of highly ( $> 4.5 \log \text{ cfu/carcass and g}$ , respectively) contaminated carcasses after bleeding. Similar trend was noted by Allen et al. (2007) whose study described significant reduction in *Campylobacter* counts after evisceration in relation to carcasses after plucking contaminated with more than  $4.2 \log \text{ cfu/g}$ .

We further showed that washing and chilling can lower *Campylobacter* carcass contamination. Nevertheless, a significant reduction of *Campylobacter* counts was observed only when comparing carcasses before washing to those after chilling ( $P < 0.05$ ). Our observations are in line with the study of Elvers et al. (2011), who reported not significant changes in *Campylobacter* counts when comparing carcasses collected before and after chilling. In contrast, the other British authors (Allen et al., 2007) presented significant reduction in *Campylobacter* counts during chilling although some carcasses in this study were subjected to longer (8 h in forced air chillier) chilling process, that is rarely encountered in modern broiler slaughterhouses.

Based on these findings, external contamination of birds at the beginning of the slaughter process as well as plucking, evisceration, washing and chilling activities seemed to have an impact on the levels of *Campylobacter* contamination. Therefore, risk factors associated with *Campylobacter* counts on carcasses collected at the following 5 sampling sites: after bleeding, after plucking, after evisceration, after washing, after chilling were elucidated.

The analyses of risk factors showed that caecal colonization level was associated with *Campylobacter* carcass contamination. Also previously, other authors indicated that the carcass contamination might be explained by *Campylobacter* concentration in intestinal samples (Reich et al., 2008; Rosenquist et al., 2006). This implies, that a reduction of the *Campylobacter* colonization level can reduce carcass contamination and consequently public health risk (Nauta et al., 2009). According to EFSA (EFSA, 2011),

fulfillment of microbiological criteria set at the critical limit of 1000 cfu/gram of neck and breast skin, can reduce public health risk of campylobacteriosis by 50 %. In the present study, all *Campylobacter* batches colonized with on average less than  $10^8$  cfu/g caecal content complied with the proposed limit of 1000 cfu/g of chilled broiler breast skin. As such more attention should be paid to reduce the *Campylobacter* colonization level which might be more attainable in the short term perspective (Hermans et al., 2014; Kittler et al., 2013b; Łaniewski et al., 2014; Neal-McKinney et al., 2014; Robyn et al., 2013) than obtaining *Campylobacter* negative status of broilers at the end of their rearing period (Hermans et al., 2011; Newell et al., 2011).

Moreover, not only colonization level of incoming birds but also external *Campylobacter* contamination (i.e. *Campylobacter* contamination of feathers) was identified as a risk factor confirming previously stated finding that the surface of incoming birds is a potential source of *Campylobacter* contamination of broiler carcasses (Berrang et al., 2000a; Seliwiorstow et al., 2015; Stern et al., 1995). External *Campylobacter* carcass contamination increases during transport, although already at the farm level before loading, birds may carry more than  $6 \log_{10}$  cfu/g *Campylobacter* on their exterior (Seliwiorstow et al., 2013; Stern et al., 1995). Type of the litter, its acidification or chemical treatment together with broilers' diet and water disposal might influence numbers of *Campylobacter* recovered from birds' exterior at the farm level. However, more research is needed to explain *Campylobacter* external contamination at the primary production. Also Implementation of longer feed withdrawal time can result in lower defecation patterns (Papa and Dickens, 1989), and consequently in slower increase of *Campylobacter* counts during transport.

Longer periods of feed withdrawal, that can be represented by a longer transport and holding time in our study, also results in lower *Campylobacter* counts on carcasses at all sampling sites. Longer feed withdrawal time allows clearance of the gastrointestinal tract and it has been reported that the majority of the weight loss in broilers during the first hours of feed withdrawal is attributed to evacuation of the gastrointestinal content (Northcutt et al., 2003). Too short withdrawal time might result in bigger and rounded birds' gastrointestinal tract full of ingest which may leak and contaminate carcasses during the slaughter process (Northcutt et al., 1997). However, if the feed withdrawal time is too long (> 14 h), tensile strength of intestinal walls decreases, being more likely to rupture during the evisceration process (Bilgili, 1988). It has been reported that the intestinal tract of birds exposed to 9-12 hours of feed withdrawal time were optimal for processing due to the fact that they were empty and reconcilable (Northcutt et al., 1997). In the present study, the time that broilers were crated ranged between 3.5 and 14 hours. Therefore, there is still a possibility to optimize the feed withdrawal time that should result in lower *Campylobacter* counts on broiler carcasses. Nevertheless, when establishing such an intervention, animal welfare as well economic aspects (e. g. weight loss, meat quality) should be considered (Delezie et al., 2007; Lyon et al., 1991).

Additionally, the statistical analyses indicated several technical aspects of the slaughter process that influence *Campylobacter* carcass contamination.

Unloading of birds with the container system (used in slaughterhouses B, C, D, E and F) was identified as a risk factor for increased *Campylobacter* counts on carcasses after bleeding. This system is based on the tilting the container and discharging live birds onto a conveyor belt (Rasschaert et al., 2007). This operation might cause intensive defecation of stressed birds resulting in the increase of the external *Campylobacter* contamination. Additionally, together with birds faces from the container floor are also discharge that possibly increases external fecal contamination of unloaded broilers. In contrast, in slaughterhouse A, an unloading system with drawers was applied, where birds were transported in plastic removable crates placed within metal modules. During unloading of those crates, they were mechanically removed from the module and transported on a conveyor belt to the hanging area where birds are placed on shackles (Rasschaert et al., 2007).

We also observed that, higher *Campylobacter* counts on carcasses collected after plucking and after washing were recovered from birds that were stunned with the electricity in comparison to those exposed to gas stunning. This difference can be explained by intensive defecation of broilers during gas stunning (Webster and Fletcher, 2001) what results in the clearance of gastrointestinal track and lower fecal contamination during slaughter.

Based on obtained results higher scalding temperature was found to reduce *Campylobacter* counts on carcasses after evisceration. Producers avoid high scalding temperatures due to quality difficulties, mainly color darkness, (Sams, 2001) although when the relatively low temperature of scalding water is applied, *Campylobacter* counts remain stable on broiler breast skin samples (Yang et al., 2001). As such, increasing of scalding temperature in the frame of *Campylobacter* counts reduction should be followed by other adaptations in the slaughter process [i. e. scalding time (Wise and Stadelman, 1961), chilling (Zhuang et al., 2013)] in order to assure satisfactory meat quality.

Furthermore, our findings indicate the importance of adjustment of a plucking machine, a vent cutter and a evisceration machine. We observed that better plucking performance (being less feathers remaining on the carcass) resulted in higher *Campylobacter* counts on carcasses after evisceration. It might be related to more pressure of the plucking fingers on carcasses resulting in the elaborated escape of the cloaca content and in higher *Campylobacter* counts on carcasses (Berrang et al., 2001). Also carcasses from batches with high percentage of broken cloaca and intestines carried higher *Campylobacter* numbers after washing and chilling, respectively. It probably related to the presence of intestinal contamination on the carcasses due to leakage of intestinal content (Berrang et al., 2004; Boysen and Rosenquist, 2009).

In conclusion, obtained results indicate that *Campylobacter* counts on carcasses at the various sampling sites were influenced by multiple factors, including aspects related to the contamination level of

incoming birds (i. e. *Campylobacter* counts in caecal content and on feathers), transport and holding time and technical characteristics of the slaughter process (e.g. unloading and stunning system, scalding water temperature, percentage of ruptured gastrointestinal packages, etc.). This confirms that effective strategies to control *Campylobacter* in broiler meat should be based on the interventions through all stages of the food chain (EFSA, 2011). Importantly, all investigated factors identified in the present study were existing variations of the routine processing practices and therefore proposed interventions can be practically and economically achievable. Although high *Campylobacter* counts on carcasses after chilling (final product) are associated with short holding and transport time, high percentage of ruptured gastrointestinal packages and high average *Campylobacter* counts in caecal content, risk factors elucidated at the other sampling sites should not be ignored because the decrease of *Campylobacter* counts at the earlier stages of the slaughter process can also result in lower contamination of chilled carcasses.

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## **GENERAL DISCUSSION**

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Campylobacteriosis is the most common form of bacterial gastroenteritis in humans (EFSA, 2014). In Belgium more than seven thousand people are diagnosed with campylobacteriosis annually (FASFC, 2012) and this seems to be only the tip of the iceberg (Havelaar et al., 2012). In most cases only milder symptoms occur and they are not reported to the health care system (Borgdorff and Motarjemi, 1997). Based on molecular epidemiological studies the main source of campylobacteriosis has been traced back to the poultry (Lévesque et al., 2013; Mughini Gras et al., 2012), and it is estimated that handling, preparation and consumption of broiler meat may account for 20 % to 30 % of human cases (EFSA, 2011a). Therefore, it is not surprising that competent authorities are seeking interventions reducing *Campylobacter* exposure caused by poultry meat to humans.

Correct response to the campylobacteriosis epidemic should be based on an integrated farm to fork approach with full commitment at all stages of the food chain, including the consumer level.

Reduction of the *Campylobacter* prevalence at the **farm level** would be the most effective approach to decrease human exposure to *Campylobacter* (Nauta et al., 2007). Since preventive measures such as vaccination, feed additives, competitive exclusion, has been shown to be not effective yet (Hermans et al., 2011; Lin, 2009) the focus for the control of *Campylobacter* at farm level is in particular oriented to biosecurity measures up to now. Biosecurity measures are considered essential to prevent flock colonisation with *Campylobacter*, but only applicable for indoor production. Still the rigorous and continuous application of strict biosecurity measures necessary to prevent *Campylobacter* entering the broiler house is hard to achieve. Newell et al. (2011) stated that application of biosecurity seems to be ineffective and thus debatable as an efficient control measure. On the other hand, most (but not all) Belgian farmers visited by us were very focused on *Salmonella* but were not aware of the bacteria called *Campylobacter*. Is it then reasonable to require (more) strict biosecurity measures without providing appropriate information about *Campylobacter* and the risk that it poses? Therefore, at first **education of producers** should be introduced in order to increase general awareness of the *Campylobacter* problem in the broiler production and the importance of preventive measures such as biosecurity.

Nevertheless, due to the complex *Campylobacter*'s ecology the implementation of tightened biosecurity will probably not rule out completely *Campylobacter* colonized broilers sent for **slaughter**. One of possibilities to reduce the risk of *Campylobacter* cross-contamination due to the processing of *Campylobacter* positive broilers is **scheduled slaughtering** that has been introduced in Iceland since October 2001 (Tustin et al., 2011). This intervention requires categorization of processed batches as either colonized or not colonized with *Campylobacter* and subjection of positive ones to special treatment (e.g. freezing). It is important to note, that such intervention is not easily applicable in

countries with relatively high *Campylobacter* prevalence at the farm level (e.g. Belgium, Ireland, Netherlands, England). Therefore, Food Safety Authority of Ireland (FSAI) recommended a **pre-harvest criterion** of 7 log<sub>10</sub> cfu/g of *Campylobacter* in pooled caecal samples (FSAI, 2011). Based on a risk assessment study, carcasses originating from flocks colonized at the level exceeding 7 log<sub>10</sub> cfu/g account for the main risk of campylobacteriosis for consumers (Nauta and Havelaar, 2008). Quantitative criteria for *Campylobacter* colonization level will also allow the application of colonization-reducing measures that might be successfully introduced in shorter perspective than the development of effective preventive measures. The drawback of the pre-harvest quantitative criterion is that it requires both a representative sampling strategy and quantification methods in order to correctly classified broiler flocks by their level of *Campylobacter* colonization.

In **Chapter 2** the comparison of different sample types (both at the farm and the slaughterhouse), used for the quantification of the *Campylobacter* colonization level, was described. Although at the farm level faecal material or boot swabs samples are easier to collect, in caecal droppings samples the highest *Campylobacter* numbers were recovered. Similarly, at the slaughterhouse level, higher *Campylobacter* counts were found in caecal content samples than in faecal material collected from transport crates. As such, it can be concluded that caecal droppings and caecal content are the most appropriate sample types for quantification of *Campylobacter* colonization level of broilers at the farm and the slaughterhouse respectively.

Since *Campylobacter* colonization can quickly spread throughout a broiler flock (van Gerwe et al., 2009), testing for *Campylobacter* should be performed as close as possible to the slaughter day to provide reliable information. Nevertheless, quantification of *Campylobacter* by culture methods is time consuming. A quicker alternative might be a qPCR method and we showed that the direct culture method and qPCR based methods are equally sensitive for *Campylobacter* enumeration in caecal content samples. Additionally, since qPCR is able to detect stressed and non-culturable cells, it is recommended to use this method for the quantification of *Campylobacter* in extensively stored caecal samples.

Nevertheless, culture and molecular methods require either time or/and laboratory facilities. Singlepath® Direct Campy Poultry test (Singlepath® test; Merck Millipore) is a rapid method (2 hours) that can be executed on-site by non-laboratory skilled personnel. It can classify flocks as being colonized with *Campylobacter* at levels of at least 7.5 log<sub>10</sub> cfu/g with a specificity and a sensitivity equal to 94 and 96 %, respectively, when analyzing caecal droppings samples and 92 and 100 % for caecal content samples (Chapter 2).

**Quantitative microbiological criteria** can be also implemented at the **slaughterhouse level**. In New Zealand, to tackle the highest incident rate in the world in 2007, the local government introduced (among other interventions) performance targets based on enumeration levels of *Campylobacter* on

poultry carcasses (Sears et al., 2011). A similar strategy is considered in the Netherlands (Swart et al., 2013), whereas in Ireland and Belgium post-harvest criterion of  $\leq 10\,000$  and  $\leq 1\,000$  cfu/g on chicken skin samples taken after chilling is recommended, respectively (FSAI, 2011; FAVV, 2013). This again confirms that **training of operators in the poultry supply chain** is required because the enumeration of *Campylobacter* on broiler meat, and not a presence/absence testing, is a key element to comprehensively evaluate the public health risk of campylobacteriosis (Nauta et al., 2009). Following this finding, Belgian *authorities* (Federal Agency for the safety of the food chain; FASFC) already since a number of years monitor *Campylobacter* in broiler meat by using also enumeration method and an FASFC action limit of 1000 cfu/g broiler neck skin is applicable.

To obtain reliable quantitative results, **methods** with high accuracy and selectivity are necessary in the **enumeration of *Campylobacter***. In **Chapter 1**, a novel plate medium for *Campylobacter* quantification on poultry meat samples were evaluated as a potential alternative to modified charcoal cefoperazone deoxycholate agar (mCCDA). When comparing the newly developed agar RAPID'*Campylobacter* agar (RAPID, Bio-Rad) with mCCDA and CampyFood agar® (CFA, bioMérieux) plates, a high level of agreement in *Campylobacter* counts obtained by these three media was found. RAPID agar was also the only medium of the three tested media that effectively suppressed the growth of the background microflora in naturally contaminated samples. As such in **Chapter 1** a possible and less labor intensive alternative for *Campylobacter* enumeration is presented, which can enable rapid and reliable *Campylobacter* quantification in poultry meat samples.

It is believed that *Campylobacter* carcass contamination during slaughter can be reduced or at least controlled by implementation of **slaughter process improvements** (Wagenaar et al., 2013; Habib et al., 2012). In New Zealand the exchange of information between producers resulted in the identification of cost-effective processing interventions that reduce the levels of *Campylobacter* on broilers (Sears et al., 2011).

In order to identify potential association between *Campylobacter* counts and batch/slaughterhouse process characteristics, *Campylobacter* quantitative data during slaughter of *Campylobacter* positive batches are required. **Chapter 3** and **chapter 5** presented *Campylobacter* counts collected along the processing line in six slaughterhouses. Obtained results indicated that external contamination of birds at the beginning of the slaughter process as well as plucking, evisceration, washing and chilling activities seemed to have an impact on the levels of *Campylobacter* contamination.

Additionally, obtained results revealed high within-batch variability among individual samples why should be taken into consideration when setting microbiological criteria for controlling *Campylobacter* in broiler slaughterhouses. Although the number of broiler carcasses after chilling contaminated with

more than 3 log<sub>10</sub> cfu/g varied between slaughterhouses we did not observe significant differences between *Campylobacter* counts at this processing step. This implies that the variability between batches explains the differences in *Campylobacter* counts. Thus in contrast to the results reported in the EFSA baseline study (EFSA, 2010a), these current research findings indicate that certain slaughterhouses are not more capable than others of controlling the *Campylobacter* counts on the carcasses. This difference in findings might be due to the fact that in the present study we not only analysed various batches in every slaughterhouse, but also six broiler carcasses per batch were sampled providing information about the within batch variability that was lacking within the EFSA baseline study.

Such variability within and in between-batches might also lead to the conclusion that slaughterhouses do not have implemented efficient *Campylobacter* control program (Jacxsens et al., 2010). To increase the ability of slaughterhouses to limit the *Campylobacter* contamination, factors influencing carcass contamination are pointed out in **Chapter 5**. As shown in this chapter, transport and holding time as well as mean *Campylobacter* counts in caecal content seem to be related to *Campylobacter* carcass contamination across the slaughter line. Moreover, in **Chapter 5** potential improvements and changes in the slaughter process were indicated. Since, all investigated factors were existing variations in the routine processing practices proposed interventions can be practical and economical achievable. Better management of the transport and holding time as well as adjustment of the equipment might be applied in a short time perspective. On the other hand it is hard to assume that slaughterhouses would change elements of their slaughter practice (e.g. unloading system, stunning procedure) without any economic support or foreseen benefits.

In addition, since physical or chemical intervention methods fail the authorization by law or do not gain consumer trust (EFSA, 2011a; MacRitchie et al., 2014), improvement of the slaughter practice in the frame of *Campylobacter* contamination control seems to be the most promising intervention at the moment. Since the flock colonization level is probably a key factor for carcass contamination, it can be interesting to process a same flock under different slaughter conditions in order to evaluate the impact of identified factors on *Campylobacter* carcasses contamination in a case-control study.

There is a general consensus that the main risk for public health accounts for *Campylobacter* positive batches (EFSA, 2011a). However, information about the *Campylobacter* carcass contamination during slaughter of negative batches after or before positive ones are still limited, especially when taking into account the fact that a contaminated slaughterhouse environment can be a vector for *Campylobacter* transmission from positive to *Campylobacter* free broilers (Corry and Atabay, 2001; Guerin et al., 2010). Also little is known about the fluctuation of *Campylobacter* counts during subsequent slaughter of *Campylobacter* positive batches. In **Chapter 4**, data describing dynamics of *Campylobacter* numbers on carcasses after selected processing stages in three broiler slaughterhouses during the consecutive



slaughter of broiler batches with a different *Campylobacter* status is presented. This information might be used for evaluation of the **logistic slaughter** utility as a potential intervention at the slaughterhouse level.

Obtained results showed that if only positive batches (with similar level of caecal colonization) were slaughtered, *Campylobacter* counts on carcasses from these batches remain stable. This implies that the main source of carcass contamination is the positive flock itself and that the sequence of slaughtering does not affect carcass contamination when positive batches are processed.

Furthermore, we showed that if day-to-day transmission occurs it is lower than 10 cfu/g. Consequently, negative batches slaughtered at the beginning of the processing day do not pose a *Campylobacter* contamination level causing a risk for human health .

However, when negative batches are processed after *Campylobacter* positive batches cross-contamination occurs. Carcasses from a *Campylobacter* negative batch processed immediately after a *Campylobacter* positive batch can be contaminated with levels of organisms similar to those from the positive batch. Additionally, in the present study after 20 minutes *Campylobacter* was still enumerated at the highest (among three visits) average level equal to 1.60, 1.48 and 2.02 log<sub>10</sub> cfu/g on carcasses after washing for slaughterhouse A, B and C, respectively.

In our opinion based on the data presented in **Chapter 4** the generalized concept that a limited number of carcasses from a negative batch became contaminated by *Campylobacter* originating from preceding positive broilers (EFSA, 2011a; Johannessen et al., 2007; Nauta et al., 2009) should be reconsidered and the risk analysis should be performed again taking these results into account.

We need to be aware that approximately 75 % of broiler meat production in Belgium is represented by meat cuts and chicken meat preparation. Recently, comprehensive studies were performed on the distribution of *Campylobacter* counts and practices contributing to *Campylobacter* contamination in Belgian **meat preparation plants** (Habib et al., 2008; Sampers et al., 2008). However, little is known about the distribution of *Campylobacter* contamination level during the cutting process. Thus, future research is required to generate quantitative data on *Campylobacter* contamination during the **cutting process** to complement the current picture of *Campylobacter* contamination in Belgian broiler production sector.

The Food Standards Agency (FSA) in UK performs a year-around survey to investigate the levels of *Campylobacter* on fresh chickens at the **retail level** (Anonymous, 2015). By publishing results, including the names of the retailers, FSA is trying to put pressure on retailers to work with poultry processors to do more to tackle *Campylobacter* numbers. Initial results indicate the effectiveness of the leak-proof packaging for poultry introduced by most retailers, which helps to reduce risks of cross contamination

in consumers' kitchens (Anonymous, 2015). Also in Iceland and New Zealand the leak-proof packaging policy has been introduced (Sears et al., 2011; Tustin et al., 2011). On the other hand, in Denmark, the local government decided to take the preventive measure based on the case-by-case risk assessment which allows to decide whether a batch poses an unacceptable risk to the consumer or not (Christensen et al., 2013).

At **consumer level**, cross-contamination and under-cooking may occur (Sampers et al., 2012). Therefore, **education activities** about food storage and food handling should be enhanced. Many efforts have been done in Iceland (Tustin et al., 2011), UK ("Don't wash raw chicken" campaign; <https://www.food.gov.uk/news-updates/campaigns/campylobacter/fsw-2014>) and Denmark (Rosenquist et al., 2009). Nevertheless, transfer of scientific knowledge to the public to change consumer behavior has often limited success up to now. Successful communication of food safety problems need to contain a message relevant to the target audience, contain reliable information that is understandable, rapidly distributed and repeated (Jacob et al., 2010).

Nevertheless, even the decrease of *Campylobacter* prevalence in broiler flocks and broiler meat does not ensure lower number of human campylobacteriosis cases (Rosenquist et al., 2009). Poultry meat is recognized as the main source of consumers' although other sources of human infection cannot be ruled out. The case-control study performed in The Netherlands indicated that next to chickens, as the major reservoir of campylobacteriosis, also cattle and environmental contamination do play a role as a source of human infection (Mughini Gras et al., 2012)

Also due to differences between countries in geographical and climate conditions as well as purchasing and eating habits, we cannot assume that interventions that were effective in certain parts of Europe/world will perform the same in Belgium. As such, there is still a lot more to be done at all stages of the Belgian poultry meat production chain to ensure that consumers can be confident in the broiler meat they purchase.

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# SUMMARY

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*Campylobacter* continues to be the leading bacterial cause of food-borne gastroenteritis with over 200 thousand reported cases in European Union. Similarly in Belgium campylobacteriosis is the most frequently reported zoonosis in humans since 2005. Although human exposure is possible via multiple pathways (food, environment, direct contact with animals), the broiler meat is the most important source of human campylobacteriosis. Therefore, potential interventions aiming at the reduction of consumer exposure to *Campylobacter* due to handling and consumption of broiler meat receives growing attention.

The **literature review** section provides not only basic information about poultry meat production and *Campylobacter* but also describes in details current knowledge on prevalence and routes of transmission for *Campylobacter* at primary production and during slaughter. Further, potential interventions at the farm level aiming at the prevention or reduction of *Campylobacter* colonization level in broilers are highlighted. There is still no readily available solution for prevention of *Campylobacter* colonization in broilers at the farm level and placing, thus, the intervention further in the food chain seems to be the more pragmatic choice in the short term perspective. The literature review section provides also an overview of potential intervention at the slaughterhouse level. Chemical decontamination and irradiation of broiler carcass is neither authorized by law nor accepted by the consumers in EU and the other alternative methods (e.g. hot water and steam treatment, crust freezing) seem to be not applicable at the moment in the Belgian poultry meat production environment. On the other hand, scheduled slaughter and performance target based on enumerated levels of *Campylobacter* on poultry carcasses have been successfully introduced in Iceland and New Zealand, respectively.

However, to set up practically achievable intervention measures in Belgium a good insight in the current situation on *Campylobacter* contamination at the slaughterhouse level is required. Therefore, the general aim of this thesis was to achieve better understanding of the *Campylobacter* occurrence during broiler slaughter.

To obtain reliable quantitative results, reliable methods with high accuracy and selectivity are necessary in the enumeration of *Campylobacter*. In **Chapter 1** *Campylobacter* counts obtained by three selective media: modified charcoal cefoperazone deoxycholate agar (mCCDA), Campy Food agar (CFA), and a novel agar RAPID' *Campylobacter* agar (RAPID) were compared on 12 artificially and 36 naturally contaminated poultry meat samples. Lin's concordance correlation coefficient and the Bland-Altman plot revealed a high level of agreement between *Campylobacter* counts when evaluating RAPID versus mCCDA and CFA plates. RAPID agar was the only medium tested that could effectively suppress the growth of the background microflora with naturally contaminated samples. Results presented in Chapter 1 indicated

that RAPID agar is highly selective media without loss of sensitivity for recovering *Campylobacter* and, therefore, it is suitable for *Campylobacter* enumeration on broiler carcasses.

Based on a risk assessment study carcasses originating from flocks colonized at the level exceeding 7 log<sub>10</sub> cfu/g account for the main risk of campylobacteriosis for consumers. This implies that implementation of this threshold for scheduling flocks going to slaughter can result in better consumer protection. As such, it is required to evaluate both sampling strategy and possible quantification methods in order to correctly classified broiler flocks by their level of *Campylobacter* colonization. The objective of **Chapter 2** was to compare different sample types (both at the farm and the slaughterhouse level) and methods (direct culture, qPCR, PMA-qPCR) applied for the quantification of the *Campylobacter* colonization level. In addition, the applicability of the lateral flow based immunoassay, Singlepath® Direct Campy Poultry test (Singlepath® test), was evaluated as a rapid method for the qualitative detection of *Campylobacter* in highly colonized broiler batches.

*Campylobacter* counts differed significantly between sample types collected at farm (caecal droppings, faeces, boot swabs) and at slaughterhouse (caecal content, faecal material from crates). Further, comparison of *Campylobacter* counts obtained by different methods (direct culture, qPCR, PMA-qPCR) in caecal droppings revealed significant differences, although this was not observed for caecal content samples. Evaluation of the Singlepath® test on caecal droppings and caecal content samples revealed acceptable level of sensitivity and specificity.

In conclusion, caecal droppings and caecal content are proposed as the most representative sample types for quantification of *Campylobacter* colonization level of broilers at farm and slaughterhouse, respectively. Direct culture and qPCR are equally sensitive for quantification of *Campylobacter* in fresh caecal content samples. Although when samples are extensively stored qPCR is preferred to direct culture. Further, Singlepath® test offers a convenient alternative method for rapid detection of *Campylobacter* in highly colonized broiler batches.

Obtaining *Campylobacter* quantitative data during slaughter of *Campylobacter* positive batches is fundamental to identify processes contributing to the carcass contamination. Therefore in **Chapter 3** *Campylobacter* contamination on broiler carcasses was quantified at seven sampling sites throughout the slaughter process. For this purpose, in four slaughterhouses samples were collected from twelve *Campylobacter* positive batches. Broilers from all visits carried high numbers of campylobacters in their caeca ( $\geq 7.9$  log<sub>10</sub> cfu/g). *Campylobacter* counts on feathers were identified as an additional source for carcass contamination. A high variability in *Campylobacter* carcass contamination on breast skin samples within batches and between batches in the same slaughterhouse was observed. In slaughterhouses A, B, C and D *Campylobacter* counts exceeded a limit of 1000 cfu/g on 50 %, 56 %, 78 % and 11 % of



carcasses after chilling, respectively. This finding indicates that certain slaughterhouses might be able to better control *Campylobacter* contamination than others. Overall, presented data in Chapter 3, focus on the descriptive analysis of *Campylobacter* counts in different slaughterhouses, different batches within a slaughterhouse and within a batch at several sampling locations.

Additionally, batch and slaughterhouse related characteristics were collected in order to identify factors associated with obtained *Campylobacter* counts (**Chapter 5**). Our results showed that reduction of *Campylobacter* colonization level and optimization of transport and holding time might result in a lower broiler carcass contamination across the slaughter line. Additionally, incorrect setting of plucking, evisceration and cloaca cutter machines, too low scalding temperature, dump based unloading system and electrical stunning were identified as risk factors associated with an increase of *Campylobacter* counts on broiler carcasses. Importantly, all investigated factors were existing variations of the routine processing practices and therefore proposed interventions can be practical and economical achievable.

There is a general consensus that the main risk for public health accounts for *Campylobacter* positive batches. However, information about the *Campylobacter* carcass contamination during slaughter of negative batches after or before positive ones is still limited. Therefore the objective of **Chapter 4** was to obtain quantitative data describing dynamics of *Campylobacter* numbers on carcasses after scalding/bleeding, plucking, evisceration and washing in three broiler slaughterhouses during the consecutive slaughter of broiler batches with a different *Campylobacter* status. As expected, slaughter of *Campylobacter* colonized batches produced contaminated carcasses. However, during consecutive slaughter of *Campylobacter* positive batches, *Campylobacter* carcass contamination remained stable with batch average counts exceeding 3.3 and 2.2 log<sub>10</sub> cfu/g on carcasses after washing in slaughterhouses A and B, respectively. Carcasses from *Campylobacter* negative batches of broiler flocks can become contaminated with *Campylobacter* (i.e. showing counts  $\geq 10$  cfu/g) when they are processed immediately after the slaughter of *Campylobacter* colonized birds. The *Campylobacter* numbers on these carcasses by cross-contamination decreased over the first 20 minutes sampling time when the negative flock was slaughtered successively to the positive flock. However, the decrease was slower than it was previously estimated in risk assessment studies. Additionally, it was observed that the *Campylobacter* counts on carcasses from negative batches were influenced by the colonization level of previously slaughtered broilers and that the evisceration process contributed the most to the cross-contamination between *Campylobacter* positive and *Campylobacter* free broilers slaughtered subsequently.

As concluded in the **general discussion** section we provided contribution toward reliable quantification of *Campylobacter* colonization level in pre-slaughter broilers and on broiler carcasses during the slaughter process. In addition, we generated extensive quantitative data on *Campylobacter* carcass

contamination during the broiler slaughter process. These data will not only be useful in the future for the estimation of the campylobacteriosis burden in Belgium but they can also significantly improve risk assessment models by replacing assumptions or experts' opinions with the experimental data. Moreover, we determined routine processing practices and batch characteristics associated with *Campylobacter* counts what may improve slaughterhouses ability to control *Campylobacter* contamination during slaughter.

We are aware about the fact that the slaughter process is only one step in the broiler meat production chain and that the control of *Campylobacter* must be based on a "farm to fork" approach, including consumer level. From a prior Belgian survey about consumer cooking habits it became clear that there is still place for improvement especially on the cross contamination aspect.

It is also worth to note that in Belgium purchasing meat cuts and chicken meat preparation is generally more popular than buying the whole carcass what implies that data describing *Campylobacter* contamination in meat preparation plants as well as during cutting is required to have a more complete picture of exposure of Belgian consumers to *Campylobacter* contamination.

Although the data suggest that fresh poultry is the main source of exposure of consumers to *Campylobacter*, other contributing factors and sources cannot be ruled out as the cause of campylobacteriosis also need attention. Additionally, when implementing interventions on a country level, the risk posed by the imported broiler meat should not be underestimated. As such even after the implementation of strategy to control *Campylobacter* contamination on Belgian farms and in the Belgian broiler slaughterhouses and further poultry meat processing, only partial decrease in the number of human cases might be expected.

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## **SAMENVATTING**

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Met meer dan 200.000 gerapporteerde gevallen in de Europese Unie blijft *Campylobacter* de meest voorkomende bacteriële oorzaak van voedselgebonden maagdarminfecties. Ook in België is campylobacteriosis sinds 2005 de meest frequent gerapporteerde zoönose. De mens wordt blootgesteld aan *Campylobacter* via verschillende wegen (voedsel, omgeving, contact met dieren), maar de belangrijkste bron voor campylobacteriosis bij de mens is braadkippenvlees. Daarom gaat steeds meer aandacht naar potentiële interventie maatregelen die tot een reductie van de humane blootstelling aan *Campylobacter* via bereiding(?) en consumptie van braadkippenvlees leiden.

In het literatuuroverzicht wordt naast informatie over pluimveevlees productie en *Campylobacter*, ook in detail de bestaande kennis omtrent de prevalentie en transmissiewegen van *Campylobacter* in de primaire sector en tijdens het slachten van braadkippen beschreven. Ook wordt aandacht besteed aan mogelijke interventie maatregelen in de primaire sector teneinde de kolonisatie van braadkippen te voorkomen of te reduceren. Op dit ogenblik zijn er geen sluitende oplossingen die de kolonisatie van de braadkippen op landbouwbedrijven kunnen verhinderen en lijken interventie maatregelen verder in de voedselketen een meer aangewezen optie op korte termijn. Er wordt dan ook een overzicht gegeven van mogelijke interventie maatregelen die in slachthuizen kunnen genomen worden. Chemische decontaminatie en bestraling van karkassen is noch wettelijk toegestaan noch aanvaard door de EU consumenten en andere alternatieve methoden zoals warm water- en stoombehandeling en 'crust freezing', zijn momenteel niet toepasbaar in de Belgische pluimveesector. Anderzijds worden logistiek slachten en performantiedoelstellingen gebaseerd op het *Campylobacter* contaminatieniveau op pluimveekarkassen met succes toegepast in respectievelijk IJsland en Nieuw Zeeland.

Om praktisch haalbare interventie maatregelen op te zetten in België is een goede kennis van de huidige *Campylobacter* contaminatie op slachthuis niveau noodzakelijk. Vandaar was de algemene doelstelling van dit doctoraal proefschrift een beter inzicht te verwerven in het voorkomen van *Campylobacter* tijdens het slachten van braadkippen.

Om betrouwbare kwantitatieve resultaten te bekomen dienen analytische methoden met een hoge nauwkeurigheid en selectiviteit gebruikt te worden voor het tellen van *Campylobacter*. In hoofdstuk 1 werden 3 selectieve media (modified charcoal cefoperazone deoxycholate agar (mCCDA), Campy Food Agar (CFA) and het nieuwe RAPID'*Campylobacter* (RAPID) agar medium) onderling vergeleken om de *Campylobacter* concentratie te bepalen in 12 artificieel en 36 natuurlijk gecontamineerde pluimveevleesmonsters. Er was een hoge graad van overeenkomst tussen de *Campylobacter* aantallen bij de vergelijking van RAPID ten opzichte van mCCDA en CFA agar. De RAPID agar was het enig medium dat in staat was om de achtergrond flora in natuurlijk gecontamineerde monsters op een afdoende wijze te onderdrukken. Resultaten laten vermoeden dat de RAPID agar een hoog selectief medium is zonder verlies aan sensitiviteit en dat dit medium bijgevolg bruikbaar is om *Campylobacter* te tellen op braadkipkarkassen.

Gebaseerd op een risicobeoordeling vormen karkassen afkomstig van tomen gekoloniseerd met minstens 7 log<sub>10</sub> kve/g cecuminhoud het hoogste risico voor campylobacteriosis bij consumenten. Dit houdt in dat het toepassen van deze drempel bij het opstellen van de slachtvolgorde kan leiden een betere bescherming van de consument. Om tomen op een correcte manier te kunnen classificeren op basis van het kolonisatieniveau is het noodzakelijk om zowel de bemonsteringsstrategie als de methoden om te kwantificeren te evalueren. De doelstelling van hoofdstuk 2 was verschillende monstertypes (zowel in pluimveebedrijven als op slachthuisniveau) en kwantitatieve methoden (cultuurmethode, qPCR, PMA-qPCR) te vergelijken. Bovendien werd de bruikbaarheid van de immunoassay Singlepath Direct Campy Poultry test (Singlepath test), geëvalueerd als snelle methode voor de kwalitatieve detectie van hoog gekoloniseerde braadkiptomen.

Het aantal *Campylobacter* verschilde significant tussen de monsters verzameld op landbouwbedrijven (cecale droppings, feces en overschoenen) en in het slachthuis (cecuminhoud en fecaal materiaal uit de transport containers). Verder bleek dat de aantallen bekomen met de verschillende methoden (cultuurmethode, qPCR, PMA-qPCR) in cecale droppings significant verschilden, terwijl dit niet het geval was voor cecuminhoud. The Singlepath test op cecale droppings en cecuminhoud leverde een aanvaardbare sensitiviteit en specificiteit op.

Als besluit kan gesteld worden dat cecale droppings en cecuminhoud de meest representatieve monstertypes zijn voor de kwantificatie van de *Campylobacter* kolonisatie van braadkiptomen respectievelijk op het landbouwbedrijf en in het slachthuis. De cultuurmethode en qPCR zijn even gevoelig voor de telling van *Campylobacter* in cecuminhoud. Wanneer de monsters langdurig bewaard worden is qPCR meer geschikt dan de cultuurmethode. Singlepath test is een geschikte alternatieve methode voor de snelle detectie van hoog gekoloniseerde braadkiptomen.

Kwantitatieve gegevens omtrent de *Campylobacter* contaminatie tijdens het slachten van *Campylobacter* positieve tomen is essentieel om processtappen te kunnen identificeren die bijdragen tot de contaminatie van de karkassen. Daartoe werd in hoofdstuk 3 de *Campylobacter* contaminatie op kippenkarkassen gekwantificeerd op 7 plaatsen in de slachtlijn. In 4 slachthuizen werden monsters genomen van 12 *Campylobacter* positieve braadkiptomen. Dieren van alle opgevolgde tomen droegen hoge aantallen campylobacters in de ceca (>7,9 log<sub>10</sub> kve/g). *Campylobacter* op de veren werd geïdentificeerd als een bijkomende bron voor de karkascontaminatie. Een hoge variatie in de karkascontaminatie werd vastgesteld, zowel binnen een toom als tussen tomen in eenzelfde slachthuis. In slachthuis A, B, C en D waren respectievelijk 50%, 56%, 78% en 11% van de karkassen na koeling met minstens 1000 kve/g gecontamineerd. Deze bevindingen suggereren dat sommige slachthuizen beter in staat zijn de *Campylobacter* contaminatie te beheersen dan andere. Hoofdstuk 3 beschrijft het kwantitatief voorkomen van *Campylobacter* in verschillende slachthuizen, verschillende tomen binnen een slachthuis en binnen een toom op verschillende locaties.

Toom en slachthuis gerelateerde gegevens werden verzameld om factoren te identificeren die gerelateerd zijn aan de bekomen *Campylobacter* aantallen (Hoofdstuk 5). Onze resultaten toonden dat een reductie van het kolonisatieniveau met *Campylobacter* en de optimalisatie van transport- en wachttijden in het slachthuis kunnen leiden tot een reductie van de karkascontaminatie doorheen het slachtproces. Het slecht afstellen van de pluk- en eviseratiemachines, een te lage temperatuur van het broeiwater, het kantelen van de transportcontainer bij het uitladen en elektrische verdoving leidden tot een verhoging van de karkascontaminatie. Alle onderzochte factoren zijn bestaande variaties van routineslachtpraktijken, waardoor de voorgestelde interventies zowel economisch als in de praktijk haalbaar zijn.

Er heerst een algemene consensus dat het risico voor de volksgezondheid uitgaat van *Campylobacter* positieve tomen. Evenwel is de informatie betreffende de contaminatie van karkassen gedurende het slachtproces van *Campylobacter* negatieve tomen geslacht voor en na positieve tomen vrij beperkt. Het doel van hoofdstuk 4 was de dynamiek van de contaminatie van karkassen na verbloeden, plukken, evisceratie en wassen in 3 slachthuizen gedurende het slachten van opeenvolgende tomen met verschillende *Campylobacter* status op te volgen. Zoals verwacht leidde het slachten van positieve tomen tot contaminatie van de karkassen. Tijdens het opeenvolgend slachten van positieve tomen bleef het contaminatieniveau echter stabiel, met een gemiddeld aantal van meer dan 3,3 en 2,2 log<sub>10</sub> kve/g op karkassen na het wassen in respectievelijk slachthuis A en B. Karkassen van negatieve tomen werden gecontamineerd (zijnde > 10 kve/g) wanneer deze geslacht werden onmiddellijk na het slachten van gekoloniseerde tomen. Het aantal *Campylobacter* op deze karkassen daalde evenwel gedurende de bemonsterde tijdsperiode van 20 min. De waargenomen daling was echter lager dan wat werd aangenomen bij vroegere risicobeoordelingsstudies. Bovendien werd vastgesteld dat de aantallen op karkassen afkomstig van negatieve tomen afhankelijk was van het kolonisatieniveau van de voorafgaande toom en dat de evisceratiestap de grootste bron voor de kruiscontaminatie was tussen positieve en negatieve tomen.

Zoals besloten wordt in algemene discussie werd een bijdrage geleverd tot een betrouwbare kwantificatie van de *Campylobacter* kolonisatie in braadkippen voor het slachten en op karkassen tijdens het slachten. Bovendien werden uitgebreide gegevens verzameld omtrent van kwantitatieve contaminatie van braadkippenkarkassen met *Campylobacter*. Deze gegevens zullen niet alleen bruikbaar zijn bij de inschatting van de ziektelast van campylobacteriose in België, maar kunnen aangewend worden om risicobeoordelingsmodellen te verbeteren door veronderstellingen en expert opinies te vervangen door experimentele gegevens. Bovendien werden routine slachtpraktijken en lotkarakteristieken geïdentificeerd die geassocieerd zijn met de *Campylobacter* contaminatie, hetgeen de slachthuizen kan bijstaan in de beheersing van de kiem tijdens het slachten.

Het slachtproces is slechts één stap in de productieketen van braadkippenvlees en de beheersing van *Campylobacter* moet gebaseerd zijn op de 'riek tot vork' benadering. Uit eerder Belgisch onderzoek naar de kookgewoontes door consumenten blijkt duidelijk dat er ruimte is voor verbetering en, zeker wat betreft het voorkomen van kruiscontaminatie. Bovendien is in België de aankoop van braadkipdelen en vleesbereidingen populairder dan deze van volledige karkassen. Dit houdt in dat resultaten omtrent de contaminatie van delen en vleesbereidingen vereist zijn om een meer totaal beeld van de blootstelling van de Belgische consument aan *Campylobacter* te bekomen.

Alhoewel gegevens laten uitschijnen dat vers pluimveevlees de belangrijkste bron is voor *Campylobacter* infecties bij de mens, kunnen andere factoren en bronnen als oorzaak van ziekte bij de mens niet uitgesloten worden. Wanneer interventies op nationaal niveau worden geïmplementeerd, mag het risico verbonden aan ingevoerd braadkippenvlees niet onderschat worden. Door het nemen van maatregelen op Belgisch niveau om de kiem te beheersen op pluimveebedrijven, in slachthuizen en in vleesverwerkingsbedrijven, kan dus slechts een partiële daling van het aantal humane gevallen verwacht worden.



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## **CURRICULUM VITAE**

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Tomasz Seliwiorstow was born on March 13th 1985 in Łódź (Poland). In 2004 he started studies at the Faculty of Biotechnology and Food Sciences at the Technical University of Łódź in Poland. Five years later he graduated as a Master of Science in Food Biotechnology.

From December 2009 he worked for one year as a Quality and Production Planning Specialist at "LESAFFRE Group" in the bakery mixes production department in Łódź (Poland).

In March 2011 he started his PhD study at the Department of Veterinary Public Health and Food Safety at Ghent University in the framework of CAMPYVAR and CAMPYTRACE projects funded by the Belgian Federal Public Services of Health, Food Chain Safety and Environment. The research was focused on the *Campylobacter* contamination of broiler carcasses during the slaughter process.

During his PhD, Tomasz Seliwiorstow has authored and co-authored several publications in the international peer-reviewed journals as well as he has presented obtained results at the multiple (inter)national conferences.

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T. Seliwiorstow, L. De Zutter

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T. Seliwiorstow, J. Baré, M. Uyttendaele, L. De Zutter

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L. De Zutter, T. Seliwiorstow

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**From Farm-to-Fork : Merck Singlepath® Direct Campy Poultry Rapid Test Kit for Direct Detection of *Campylobacter* spp. in Fresh Caecal Samples From Live Chicken**

*L. John, J. Slaghuis, M. Wadl, M. Wagner, T. Seliwiorstow, J. Baré, M. Uyttendaele, L. De Zutter, C. Lindhardt*

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**From Farm-to-Fork: Merck *Singlepath*® Direct Campy Poultry Rapid Test Kit for Farm-Based Direct Detection of *Campylobacter* spp. in Faecal and Caecal Samples from Live Chicken**

*L. John, J. Slaghuis, M. Wadl, G. Schalleger, M. Glatzl, B. Stessl, M. Wagner, T. Pölzler, T. Seliwiorstow, L. De Zutter, C. Lindhardt*

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